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(54) Title: DEVELOPMENT OF DNA PROBES AND IMMUNOLOGICAL REAGENTS SPECIFIC FOR CELL SURFACE-EXPRESSED MOLECULES AND TRANSFORMATION-ASSOCIATED GENES			
(57) Abstract <p>This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not the other. This invention also provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein. This invention provides a method to prepare a hybridoma cell line which specifically recognizes and binds to a tumor associated antigen associated with a neoplastic, human cell. This invention also provides a method of preparing DNA encoding a cell surface antigen associated with a neoplastic, human cell. This invention further provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Carcinoma Tumor Antigen Gene-1. This invention also provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1. This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-2. Finally, this invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1.</p>			

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DEVELOPMENT OF DNA PROBES AND IMMUNOLOGICAL  
REAGENTS SPECIFIC FOR CELL SURFACE-EXPRESSED  
MOLECULES AND TRANSFORMATION-ASSOCIATED GENES

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The invention disclosed herein was made with Government support under NIH Grants CA 35675 and CA 43208 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

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Background of the Invention

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Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

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Full bibliographic citation for these references may be found at the end of each series of experiments.

25

The classical method for developing monoclonal antibodies specific for cell-surface molecules involves repeated injections of mice with either intact cells or cell membrane preparations derived from the desired target cells. The injections are followed by the removal of mouse spleen cells and fusion of these cells to a myeloma partner [reviewed in (1-3)]. This approach has resulted

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in the production of monoclonal antibodies that react with a number of surface-expressed molecules of potential interest, including cell-surface growth factor receptors and tumor-associated antigens. However, the procedure is generally inefficient and requires screening of a large

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number of hybridomas for production of the appropriate monoclonal antibodies [reviewed in (1-6)].

DNA transfection procedures have been used to transfer

-2-

human genes into heterologous cells, such as mouse NIH 3T3 cells [reviewed in 7-10]]. When NIH 3T3 cells have been used as the recipient for DNA transfection, this approach has not been successful in identifying dominant-acting transforming or tumor-inducing genes from a majority (approximately 85%) of human tumors or human tumor cell lines (7-10). In most studies that use NIH 3T3 cells, even when a dominant-acting oncogene was identified, it often represented a member of the ras oncogene family or a modified cellular gene (7-10). A recently developed cloned rat embryo fibroblast cell line, CREF-Trans 6, has proven useful in identifying putative novel oncogenes not detected in NIH 3T3 cells (11). Cotransfection of CREF-Trans 6 cells with high-molecular-weight DNA from the LNCaP human prostatic carcinoma cell line and the selectable neomycin resistance gene (pSV2neo), followed by selection for resistance to G418 and injection into nude mice, resulted in tumor formation (11). In contrast, when the same DNA sources were used with NIH 3T3 cells, no tumors developed in nude mice given an injection of neomycin-resistant (G418) cotransfected NIH 3T3 cells (11).

Applicants conducted the current experiments to determine if DNA transfection combined with an immunologic masking tactic could be used to efficiently generate hybridomas that secrete monoclonal antibodies reacting with cell-surface molecules expressed on genetically altered cells. Applicants demonstrate the feasibility of this approach, called surface-epitope masking (SEM). Applicants used DNA transfection and the SEM procedure in an attempt to produce hybridomas secreting monoclonal antibodies that reacted with surface epitopes located on typical multidrug-resistant (MDR) cells and human prostatic carcinoma cells. These results indicate that DNA



-3-

transfection in conjunction with SEM can be used to generate hybridomas producing monoclonal antibodies that can react with surface-expressed molecules encoded by both known and unknown genes.

Summary of the Invention

- 5 This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not the other comprises a) generating antiserum against a cell type which does not express the cell surface-expressed protein; b) coating another cell type which  
10 expresses the cell surface-expressed protein with the antiserum generated; c) injecting the antiserum-coated cells into suitable hosts; d) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; e) removing spleens from the hosts so  
15 identified; f) preparing from the spleens so removed hybridomas; and g) recovering therefrom a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein.
- 20 This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-expressed protein which expresses on the surface of one cell type but not the other comprising: a) generating antiserum against a cell type which does not express the  
25 cell surface-expressed protein; b) coating another cell type which expresses the cell surface-expressed protein with the antiserum generated; c) injecting the antiserum-coated cells into suitable hosts; d) screening the resulting hosts to identify hosts which produce serum  
30 reactive with the coated cell; e) removing spleens from the hosts so identified; f) isolating B-lymphocytes from the removed spleen; g) preparing DNA from plasma cells to generate combinatorial phage cDNA library which contains different clones; and h) contacting the clones in the  
35 library with the coated cells from step (b), the binding

-5-

of the coated cells with a clone indicating the protein expressed by the clone capable of binding to the cell surface-expressed protein.

5 This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not  
10 the other comprises: a) generating antiserum against a cell type which does not express the cell surface-expressed protein; b) coating another cell type which expresses the cell surface-expressed protein with the antiserum generated; c) contacting the antiserum-coated  
15 cells with suitable immunoresponsive cells capable of being stimulated to produce antibodies; d) preparing immunoresponsive cells to produce hybridomas; and e) isolating hybridomas which produce antibodies reactive with the coated cell, thereby preparing hybridoma cell  
20 lines which produce antibodies capable of specifically binding to a cell surface-expressed protein.

This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein  
25 comprises: a) generating antiserum against a cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule encoding the cell surface-expressed protein to express the cell surface-expressed protein into the cell; c) selecting cells which  
30 express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) injecting the antiserum-coated cells into suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g)  
35 removing spleens from the hosts so identified; h)

-6-

preparing from the spleens so removed hybridomas; and i) recovering therefrom a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein.

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This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-expressed protein comprising: a) generating antiserum against a cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule which encodes the cell surface-expressed protein to express the cell surface-expressed protein into the cell; c) selecting cells which express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) injecting the antiserum-coated cells into suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) isolating B-lymphocytes from the removed spleen; i) preparing DNA from B-lymphocytes to generate combinatorial phage cDNA library which contains different clones; and j) contacting the clones in the library with the coated cells from step (b), the binding of the coated cells with a clone indicating the protein expressed by the clone capable of binding to the cell surface-expressed protein.

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This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not the other comprises: a) generating antiserum against a cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule which encodes the cell surface-expressed protein to express the

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-7-

cell surface-expressed protein into the cell; c) selecting cells which express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) contacting the antiserum-coated  
5 cells with suitable immunoresponsive cells capable of being stimulated to produce antibodies; f) preparing immunoresponsive cells to produce hybridomas; and g) isolating hybridomas which produce antibodies reactive with the coated cell, thereby preparing hybridoma cell  
10 lines which produce antibodies capable of specifically binding to a cell surface-expressed protein.

This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of  
15 specifically binding to a cell surface-expressed protein comprises: a) introducing a DNA molecule which encodes a cell surface-expressed protein and a second DNA molecule which encodes a selectable or identifiable trait into an established cell line; b) selecting transfected cells  
20 which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum generated against the established cell line; e) injecting the antiserum-coated cells into the suitable hosts; f)  
25 screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) preparing from the spleens so removed hybridomas; and i) recovering therefrom a hybridoma cell line which produces an  
30 antibody capable of specifically binding to a cell surface-expressed protein.

This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-  
35 expressed protein comprising: a) introducing a DNA

- 8 -

molecule which encodes a cell surface-expressed protein and a second DNA molecule which encodes a selectable or identifiable trait into an established cell line; b) selecting transfected cells which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum generated against the established cell line; e) injecting the antiserum-coated cells into the suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) isolating B-lymphocytes from the removed spleen; i) preparing DNA from B-lymphocytes to generate combinatorial phage cDNA library which contains different clones; and j) contacting the clones in the library with the coated cells from step (b), the binding of the coated cells with a clone indicating the protein expressed by the clone capable of binding to the cell surface-expressed protein.

This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein comprises a) introducing a DNA molecule which encodes a cell surface-expressed protein and a second DNA molecule which encodes a selectable or identifiable trait into an established cell line; b) selecting transfected cells which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum generated against the established cell line; e) contacting the antiserum-coated cells with suitable immunoresponsive cells capable of being stimulated to produce antibodies; f) preparing immunoresponsive cells to produce hybridomas; and g) isolating hybridomas which

-9-

produce antibodies reactive with the coated cell of step (d), thereby preparing hybridoma cell lines which produce antibodies capable of specifically binding to a cell surface-expressed protein.

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This invention provides a method for preparing a hybridoma cell line which produces an antibody which specifically recognizes and binds to a tumor associated antigen associated with a neoplastic, human cell which

10 comprises: a) cotransfecting the CREF-Trans 6 cell line (ATCC Accession No. CRL 10584) with DNA isolated from a neoplastic, human cell and DNA which encodes a selectable or identifiable trait; b) selecting transfected cells which express the selectable or identifiable trait;

15 c) recovering the transfected cells so selected; d) injecting the transfected cells so recovered into a suitable first murine host; e) maintaining the resulting first murine host for a period of time effective to induce the injected transfected cells to form a tumor in

20 the first murine host; f) isolating the resulting tumor from the first murine host; g) obtaining tumor cells from the tumor so isolated; h) coating the tumor cells so obtained with an antiserum generated against the established non-human, non-tumorigenic cell line; i)

25 injecting the antiserum-coated cells into the suitable second hosts; j) screening the resulting second hosts to identify hosts which produce serum reactive with the neoplastic, human cell; k) removing spleens from the second hosts so identified; l) preparing from the spleens

30 so removed hybridomas; and m) recovering therefrom a hybridoma cell line which produces an antibody which specifically recognizes and binds to the cell surface antigen.

35 This invention provides a method of preparing DNA

-10-

encoding a cell surface antigen associated with a neoplastic, human cell which comprises: a) cotransfecting CREF-Trans 6 cell line with DNA isolated from a neoplastic human cell and DNA encoding a selectable or identifiable trait; b) selecting transfected cells which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) injecting the transfected cells so recovered into a suitable first murine host; e) maintaining the resulting first murine host for a period of time effective to induce the injected transfected cells to form a tumor in the first murine host; f) isolating the resulting tumor from the first murine host; g) obtaining tumor cells from the tumor so isolated; and h) recovering DNA encoding the cell surface antigen associated with the neoplastic human cell from the tumor cells so obtained.

This invention further provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Carcinoma Tumor Antigen Gene-1. This invention also provides an isolated mammalian nucleic acid molecules having the sequence of Prostate Tumor Inducing Gene-1. This invention provides an isolated mammalian nucleic acid molecules having the sequence of Prostate Tumor Inducing Gene-2. Finally, this invention provides an isolated mammalian nucleic acid molecules having the sequence of Prostate Tumor Inducing Gene-3.



-11-

Brief Description of the FiguresFigure 1

5 Flow chart for SEM strategy. This procedure involves the production of anti-CREF-Trans 6 polyclonal antibodies, which are used to block rat antigenic epitopes on transfected CREF-Trans 6 prior to injection into animals. The strategy results in the production of immune spleen cells that react with transfected surface-expressed antigens on CREF-Trans 6 cells. Spleen cells are then fused with NS1 murine myeloma cells, producing hybridomas secreting monoclonal antibodies specific for antigens expressed on the cell surface of transfected CREF-Trans 6 cells.

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Figure 2

20 Reactivity toward MDR CREF-Trans 6 cells subject to SEM produced MDR monoclonal antibodies. CREF-Trans 6 cells were transfected with a human MDR-1 gene and MDR clones resistant to colchicine were isolated. The SEM approach was used to generate independent hybridomas (MDR 2.3, MDR 3.0, MDR 8.12 and MDR 9.7) that secreted monoclonal antibodies reacting with surface epitopes of the MDR transporter expressed in CREF-Trans 6:MDR A1 cells but not untransfected non-MDR CREF-Trans 6 parental cells. The SEM-derived monoclonal antibodies were also tested for reactivity with three independently derived CREF-Trans 6 MDR clones. CREF-Trans 6:MDR C3, CREF-Trans 6:MDR D2 and CREF-Trans 6:MDR F4. Samples

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-12-

were analyzed by FACS, and results are expressed as mean fluorescence intensity units. Replicate samples varied by <10% and replicate studies varied by <20%.

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Figure 3

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Reactivity toward MCF7 and MDR MCF7 cells of human leukocyte antigen class 1 monoclonal antibodies and MDR monoclonal antibodies produced using CREF-Trans 6:MDR A1 cells and the SEM procedure. MCF7 and MCF7 CL4 cells are non-MDR cells. MCF7 CL4:MDR I, MCF7 CL4:MDR II, and MCF7 CL4:MDR III are three independent MDR MCF7 CL4 subclones. Fluorescence baseline was determined using irrelevant isotype-matched antibody and goat anti-mouse immunoglobulin G conjugated to fluoresceine isothiocyanate. Samples were analyzed by FACS and results are expressed as mean fluorescence intensity units. Replicate samples varied by  $\leq 10\%$  and replicate studies varied by  $\leq 20\%$ .

Figure 4

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Reactivity toward untransfected CREF-Trans 6, LNCaP DNA-transfected CREF-Trans 6 and human prostatic carcinoma cell lines of SEM-derived monoclonal antibodies. Pro 1.1, 1.2, 1.3, 1.4 and 1.5 are monoclonal antibodies produced by independent hybridomas generated by fusing spleen cells from mice immunized with LNCaP DNA-transfected, tumor-derived CREF-Trans 6 cells coated with anti-CREF-Trans 6 polyclonal antibodies, CREF-Trans 6:4 NMT, with NS1 murine myeloma cells. The cells

-13-

analyzed were parental CREF-Trans 6-4 NMT (primary LNCaP DNA-transfected, tumor derived transfectant derived from tumor), CREF-Trans 6:4-7 NMT (secondary CREF-Trans 6:4 NMT DNA-transfected, tumor-derived transfectant derived from tumor), and human prostatic carcinoma cell lines LNCaP, DU-145 and PC-3. Samples were analyzed by FACS, and results are expressed as mean fluorescence intensity units. Replicate samples varied by <10% and replicate studies varied by <15%.

Figure 5

Expression of putative human prostatic carcinoma encoded polypeptides in LNCaP DNA-transfected tumor-derived CREF-Trans 6 cells and LNCaP and DU-145 human prostatic carcinoma cells. Labeled cell lysates from the cell lines were immunoprecipitated with monoclonal antibody Pro 1.4. Molecular weight size markers are indicated on the left side of the figure. Experimental details can be found in the Materials and Methods section and Duigou et al. (20).

Figure 6

Differential display (DD) of CREF-Trans 6 and CREF-Trans 6:4 NMT mRNAs. DD was performed with a 24 oligomer (5'-) and a 14 oligomer (3'-) with sequences shown in materials and methods. The arrow indicates the PTI-1 band appearing only in mRNA from CREF-Trans 6:4 NMT, but not in CREF-Trans 6 cells. The length of this PTI-1 DNA fragment is 214 bp.

-14-

Figure 7

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Expression of PTI-1 in normal and tumor cell lines. RNAs from CREF-Trans 6, CREF-Trans 6:4 NMT and various normal and tumor-derived human cell lines were transferred to nylon membranes and probed with a [<sup>32</sup>P]-labeled 279 bp PTI-1 DNA fragment (317 to 596 bp, between primers A and L, Fig. 8). Membranes were stripped and reprobbed with a [<sup>32</sup>P]-labeled GAPDH gene.

Figure 8

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Peptide and DNA sequence of the PTI-1 gene and comparison with the human EF-1 $\alpha$  gene. (A) Peptide and DNA sequence of PTI-1. The 5'- and 3'-non-translation region of the PTI-1 gene is in small letters and the PTI-1 open-reading frame is in capital letters. Squared amino acids are mutated amino acids in the PTI-1 gene resulting from single-base mutations (underlined bases). The sequences underlined in the 5'-non-translated regions are the L and A primers, used in Figures 7 and 9. (B) Peptide comparison of EF-1 $\alpha$  and PTI-1. Peptide for EF-1 $\alpha$  is indicated in (E) and the peptide for PTI-1 is indicated in (P). The underlined region (67 amino acids) of EF-1 $\alpha$  indicates the amino acids missing in the PTI-1 gene. Bold letters (with a \*) indicate the mutated amino acids in the PTI-1 peptide. (C) Differences in amino acids, codons and nucleotides between EF-1 $\alpha$  and PTI-1. Six single-base mutations give rise to specific amino acid changes in the PTI-1 gene. In the column of amino

-15-

acids, the numbers in parentheses refer to the position of the amino acid in the peptide and codon refers to the sequence of three nucleotides encoding the specific amino acid. The specific nucleotide change is also indicated.

Figure 9

RT-PCR analysis of PTI-1, PSA and GAPDH expression in cell lines and tissue samples of normal prostate, BPH and prostate carcinoma. RT-PCR of PTI-1 uses two primers consisting of a pair of 20-oligomers: primer L with the sequences 5'-GAGTCTGAATAGGGCGACTT-3' (sense orientation); and primer A with the sequence 5'-AGTCAGTACAGCTAGATGCC-3' (antisense orientation) (both underlined in Fig. 8). RT-PCR of PSA uses the primers (A) 5'-AGACACAGGCCAGGTATTTTCAGGTC-3' and (B) 5'-CACGATGGTGTCTTGTATCCACTTC-3'. RT-PCR of GAPDH uses a pair of primers with the sequences (I) (5'-TCTTACTCCTTGGAGGCCATG-3') and P (II) (5'-CGTCTTCACCACCATGGAGAA-3'). The PCR amplified products were blotted on nylon membranes and probed with a [<sup>32</sup>P]-labeled 279 bp DNA fragment of PTI-1, PSA or GAPDH, respectively.

Figure 10

In vitro translation of the PTI-1 gene. Lane CAT is the in vitro translation of the chloramphenicol acetyltransferase gene ( $M_r = 24$  kDa), used as a positive control. Lane PTI-1 contains the translated products of the PTI-1 gene. Rainbow

-16-

protein standards (Amersham Life Science) were used to determine the sizes of the in vitro translated products.

5     Figure 11

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Expression of PTI-1 in CREF cells transformed by different oncogenes. Northern hybridization analysis of RNA isolated from: CREF-Trans 6; LNCaP DNA transfected nude mouse tumor-derived CREF-Trans 6 cells (CREF-Trans 6:4 NMT); LNCaP; CREF cells transformed by a mutant of type 5 adenovirus (CREF-H5hr1/A2); CREF cells transformed by a dexamethasone inducible (mouse mammary tumor virus (MMTV) promoter) wild type 5 adenovirus transforming E1A gene (CREF/MMTV-Ad5E1A) in the absence of DEX (-DEX) normal cellular phenotype, in the presence of DEX (+DEX) Ad5 E1A expressed and cells are transformed; CREF cells transformed by Ha-ras oncogene (CREF-ras); CREF cells transformed by v-src oncogene (CREF-src); and CREF cells transformed by oncogenic human papilloma virus type 51 (CREF-HPV-51). Blots probed with a <sup>32</sup>P-labeled PTI-1 gene probe, then stripped and reprobed with a <sup>32</sup>P-labeled GAPDH gene probe.

30     Figure 12

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Reactivity of Br-car (breast carcinoma) monoclonal antibodies (MAbs) prepared by the surface epitope masking (SEM) technique toward fresh-frozen sections of human cancers. Sections were prepared from patients with metastatic melanoma (A),

-17-

5 small cell lung carcinoma (B) and breast  
carcinomas (C and D). Reactivity was  
determined using immunohistochemical  
techniques with MAbs prepared using the  
SEM procedure with nude mouse tumor-  
derived CREF-Trans 6 cells transfected  
with DNA from the human breast carcinoma  
cell line T47D, CREF-Trans 6:T47D NMT.  
10 Reactivity is only apparent in the two  
human breast carcinoma sectioned patient  
samples.

Figure 13 Nucleic acid sequence of PTI-2

15 Figure 14 Nucleic acid sequence of PTI-3

Figure 15 Nucleic acid sequence of PCTA-1

20 Figure 16 Immunofluorescence staining and  
immunoprecipitation analysis of secreted  
and cellular PCTA-1. Live cells were  
incubated with MoAb Pro 1.5 and analyzed  
by fluorescence microscopy (A, B, C).  
Cells were labeled with <sup>35</sup>S-methionine and  
25 secreted and cellular PCTA-1 levels were  
determined by immunoprecipitation with the  
Pro 1.5 MoAb (D).

30 Figure 17 Immunohistochemical detection of PCTA-1  
and prostate specific antigen (PSA) in  
prostate tissue sections. Benign prostate  
[PCTA-1 (A), PSA (E)], prostatic  
intraepithelial neoplasia (PIN) [PCTA-1  
(C), PSA (F)] and invasive prostate  
35 carcinoma [PCTA-1 (C and D), PSA (G and

-18-

H)]. Original magnifications: A, B, D, E, F and H = 250X; C and G = 100X.

Figure 18

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Predicted amino acid sequence of PCTA-1 and alignment of PCTA-1 with other galactose-binding lectin (galectin) proteins. The predicted amino acid sequence is shown below the nucleotide sequence (A). Comparison of PCTA-1 with sequences of the  $M_r$  14,000 and  $M_r$  29,000 to 31,000 galectins from eel, chicken, mouse, rat, bovine and human galectins (B). \*, amino acids unique to PCTA-1; \_, amino acids shared by PCTA-1 and mouse-L34, human galectin-3- L29 and human- L31; o, amino acids shared by PCTA-1 and the  $M_r$  14,000 and  $M_r$  29,000 to 31,000 galectins from different species.

20 Figure 19

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RT-PCR analysis of PCTA-1, PSA and GAPDH expression in cell lines and tissue samples of normal prostate, BPH, and prostate carcinoma. RT-PCR of PCTA-1 uses two 20-mers with the sequences 5'-AAGCTGACGCCTCATTTGCA-3' and 5'-AACCACCAATGGAAGTGGGT-3'. RT-PCR of PSA uses two primers: primer A, 5'-AGACACAGGCCAGGTATTTTCAGGTC-3'; and primer B, 5'-CACGATGGTGTCCTTGAT CCACTTC-3'. RT-PCR of GAPDH uses a pair of primers with the sequences 5'-TCTTACTCCTTGGAGGCCATG-3' and 5'-CGTCTTCACCACCATGGAGAA-3'. The PCR-amplified products were blotted on nylon membranes and probed with a



-19-

<sup>32</sup>P-labeled DNA fragment of PCTA-1, PSA,  
or GAPDH, respectively.

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-20-

Detailed Description of the Invention

Throughout this application, the following standard abbreviations are used throughout the specification to  
5 indicate specific nucleotides:

C=cytosine	A=adenosine
T=thymidine	G=guanosine

10 This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not the other comprises: a) generating antiserum against a cell type which does not express the cell surface-expressed protein; b) coating another cell type which  
15 expresses the cell surface-expressed protein with the antiserum generated; c) injecting the antiserum-coated cells into suitable hosts; d) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; e) removing spleens from the hosts so identified; f) preparing from the spleens so removed hybridomas; and g) recovering therefrom a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein.  
20

25 This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-expressed protein which expresses on the surface of one cell type but not the other comprising: a) generating antiserum against a cell type which does not express the cell surface-expressed protein; b) coating another cell type which expresses the cell surface-expressed protein with the antiserum generated; c) injecting the antiserum-coated cells into suitable hosts; d) screening the  
30 resulting hosts to identify hosts which produce serum  
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-21-

reactive with the coated cell; e) removing spleens from the hosts so identified; f) isolating B-lymphocytes from the removed spleen; g) preparing DNA from plasma cells to generate combinatorial phage cDNA library which contains  
5 different clones; and h) contacting the clones in the library with the coated cells from step (b), the binding of the coated cells with a clone indicating the protein expressed by the clone capable of binding to the cell surface-expressed protein.

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This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not  
15 the other comprises: a) generating antiserum against a cell type which does not express the cell surface-expressed protein; b) coating another cell type which expresses the cell surface-expressed protein with the antiserum generated; c) contacting the antiserum-coated  
20 cells with suitable immunoresponsive cells capable of being stimulated to produce antibodies; d) preparing immunoresponsive cells to produce hybridomas; and e) isolating hybridomas which produce antibodies reactive with the coated cell, thereby preparing hybridoma cell  
25 lines which produce antibodies capable of specifically binding to a cell surface-expressed protein.

This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of  
30 specifically binding to a cell surface-expressed protein comprises: a) generating antiserum against a cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule encoding the cell surface-expressed protein to express the cell surface-  
35 expressed protein into the cell; c) selecting cells which

-22-

express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) injecting the antiserum-coated cells into suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) preparing from the spleens so removed hybridomas; and i) recovering therefrom a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein.

This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-expressed protein comprising: a) generating antiserum against a cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule which encodes the cell surface-expressed protein to express the cell surface-expressed protein into the cell; c) selecting cells which express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) injecting the antiserum-coated cells into suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) isolating B-lymphocytes from the removed spleen; i) preparing DNA from B-lymphocytes to generate combinatorial phage cDNA library which contains different clones; and j) contacting the clones in the library with the coated cells from step (b), the binding of the coated cells with a clone indicating the protein expressed by the clone capable of binding to the cell surface-expressed protein.

This invention provides a method for preparing a

-23-

hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein which expresses on the surface of one cell type but not the other comprises: a) generating antiserum against a  
5 cell which normally does not express the cell surface-expressed protein; b) introducing a DNA molecule which encodes the cell surface-expressed protein to express the cell surface-expressed protein into the cell; c)  
10 selecting cells which express the cell surface-expressed protein; d) coating the selected cells with the antiserum generated in step a; e) contacting the antiserum-coated cells with suitable immunoresponsive cells capable of being stimulated to produce antibodies; f) preparing  
15 immunoresponsive cells to produce hybridomas; and g) isolating hybridomas which produce antibodies reactive with the coated cell, thereby preparing hybridoma cell lines which produce antibodies capable of specifically binding to a cell surface-expressed protein.

20 This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein comprises: a) introducing a DNA molecule which encodes a cell surface-expressed protein and a second DNA molecule  
25 which encodes a selectable or identifiable trait into an established cell line; b) selecting transfected cells which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum  
30 generated against the established cell line; e) injecting the antiserum-coated cells into the suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) preparing from  
35 the spleens so removed hybridomas; and i) recovering

-24-

therefrom a hybridoma cell line which produces an antibody capable of specifically binding to a cell surface-expressed protein.

- 5 This invention provides a method for isolating DNA coding for a protein capable of binding to the cell surface-expressed protein comprising: a) introducing a DNA molecule which encodes a cell surface-expressed protein and a second DNA molecule which encodes a selectable or  
10 identifiable trait into an established cell line; b) selecting transfected cells which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum generated against the  
15 established cell line; e) injecting the antiserum-coated cells into the suitable hosts; f) screening the resulting hosts to identify hosts which produce serum reactive with the coated cell; g) removing spleens from the hosts so identified; h) isolating B-lymphocytes from the removed  
20 spleen; i) preparing DNA from B-lymphocytes to generate combinatorial phage cDNA library which contains different clones; and j) contacting the clones in the library with the coated cells from step (b), the binding of the coated cells with a clone indicating the protein expressed by  
25 the clone capable of binding to the cell surface-expressed protein.

- This invention provides a method for preparing a hybridoma cell line which produces an antibody capable of  
30 specifically binding to a cell surface-expressed protein comprises a) introducing a DNA molecule which encodes a cell surface-expressed protein and a second DNA molecule which encodes a selectable or identifiable trait into an established cell line; b) selecting transfected cells  
35 which express the selectable or identifiable trait; c)

-25-

recovering the transfected cells so selected; d) coating the selected cells so recovered with an antiserum generated against the established cell line; e) contacting the antiserum-coated cells with suitable  
5 immunoresponsive cells capable of being stimulated to produce antibodies; f) preparing immunoresponsive cells to produce hybridomas; and g) isolating hybridomas which produce antibodies reactive with the coated cell of step (d), thereby preparing hybridoma cell lines which produce  
10 antibodies capable of specifically binding to a cell surface-expressed protein.

In an embodiment, the DNA molecules are introduced into the established cell line by cotransfection.

15

In another embodiment, the DNA molecule encoding a cell surface-expressed protein is a expression vector.

In another embodiment, the cell surface-expressed protein  
20 is the P-glycoprotein.

In another embodiment, the cell surface-expressed protein is a cytokine receptor.

25 In a further embodiment, the cytokine receptor is a interferon-alpha receptor.

In still another embodiment, the cytokine receptor is a  
interferon-gamma receptor.

30

In yet another embodiment, the cell surface-expressed protein is a tumor associated antigen.

In a still further embodiment, the second DNA molecule  
35 encoding the selectable or identifiable trait is plasmid

-26-

DNA.

In another embodiment, the plasmid DNA encodes resistance to an antibiotic.

5

In still another embodiment, the plasmid DNA comprises pSV2-Neo.

10

In another embodiment, the cell line is the CREF-Trans 6 cell line (ATCC Accession No. CRL 10584).

15

This invention provides a method for preparing a hybridoma cell line which produces an antibody which specifically recognizes and binds to a tumor associated antigen associated with a neoplastic, human cell which comprises: a) cotransfecting the CREF-Trans 6 cell line (ATCC Accession No. CRL 10584) with DNA isolated from a neoplastic, human cell and DNA which encodes a selectable or identifiable trait; b) selecting transfected cells

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which express the selectable or identifiable trait; c) recovering the transfected cells so selected; d) injecting the transfected cells so recovered into a suitable first murine host; e) maintaining the resulting first murine host for a period of time effective to induce the injected transfected cells to form a tumor in the first murine host; f) isolating the resulting tumor from the first murine host; g) obtaining tumor cells from the tumor so isolated; h) coating the tumor cells so obtained with an antiserum generated against the established non-human, non-tumorigenic cell line; i) injecting the antiserum-coated cells into the suitable second hosts; j) screening the resulting second hosts to identify hosts which produce serum reactive with the neoplastic, human cell; k) removing spleens from the second hosts so identified; l) preparing from the spleens so removed



-27-

hybridomas; and m) recovering therefrom a hybridoma cell line which produces an antibody which specifically recognizes and binds to the cell surface antigen.

- 5 In an embodiment, the neoplastic, human cell is a benign cell. In another embodiment, the neoplastic, human cell is a metastatic cell. In a separate embodiment, the neoplastic, human cell is a human prostatic carcinoma cell derived from cell line LNCaP (ATCC No. CRL 1740).
- 10 In another embodiment, the cell is a human breast carcinoma cell derived from cell line T47D (ATCC No. HTB 133).

- In an embodiment, the suitable second host is a murine host.
- 15 In another embodiment, the suitable second host is a non-human primate host.

- This invention provides a method of preparing DNA encoding a cell surface antigen associated with a neoplastic, human cell which comprises: a) cotransfecting
- 20 CREF-Trans 6 cell line with DNA isolated from a neoplastic human cell and DNA encoding a selectable or identifiable trait; b) selecting transfected cells which express the selectable or identifiable trait; c)
- 25 recovering the transfected cells so selected; d) injecting the transfected cells so recovered into a suitable first murine host; e) maintaining the resulting first murine host for a period of time effective to induce the injected transfected cells to form a tumor in
- 30 the first murine host; f) isolating the resulting tumor from the first murine host; g) obtaining tumor cells from the tumor so isolated; and h) recovering the DNA encoding the cell surface antigen associated with the neoplastic human cell from the tumor cells so obtained. The DNA
- 35 molecule containing the sequence for the cell surface

-28-

antigen associated with the neoplastic human cell may be further isolated.

5 In an embodiment, the neoplastic, human cell is a benign tumor cell. In another embodiment, the neoplastic, human cell is a metastatic cell. In a separate embodiment, the neoplastic, human cell is a human prostatic carcinoma cell derived from cell line LNCaP (ATCC No. CRL 1740). In another embodiment, the neoplastic, human cell is a  
10 human breast carcinoma cell derived from cell line T47D (ATCC No. HTB133). In another embodiment, the neoplastic, human cell is a human glioblastoma multiform (stage IV astrocytoma) cell derived from a primary tumor. In a further embodiment, the neoplastic, human cell is a  
15 patient-derived metastatic colon carcinoma.

In a separate embodiment, the DNA encoding the selectable or identifiable trait is plasmid DNA encoding resistance to an antibiotic. In a further embodiment, the plasmid  
20 DNA comprises pSV2-Neo and the selection is by the antibiotic G418.

The cell surface antigen of the above-described method includes but not limited to a tumor associated antigen,  
25 a growth factor receptor, a viral-encoded surface-expressed antigen, a oncogene product, a surface epitope, a membrane protein which mediates classical multi-drug resistance, a membrane protein which mediates atypical multi-drug resistance, an antigen which mediates a  
30 tumorigenic phenotype, an antigen which mediates a metastatic phenotype, an antigen which suppresses a tumorigenic phenotype, an antigen which suppresses a metastatic phenotype and cytokine receptors including the human interferon  $\alpha$  and interferon  $\gamma$  receptors.

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-29-

In an embodiment, the cell surface antigen is an antigen which is recognized by a specific immunological effector cell. In a further embodiment, the specific immunological effector cell is a T-cell.

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In a separate embodiment, the cell surface antigen is an antigen which is recognized by a non-specific immunological effector cell. In a further embodiment, the non-specific immunological effector cell is a macrophage cell. In a still further embodiment, the non-specific immunological effector cell is a natural killer cell.

10

This invention provides the DNA prepared the above-described method. This invention also provides nucleic acid probes hybridizable with the isolated DNA molecule. The nucleic acid probe may be DNA or RNA. In an embodiment, the nucleic acid probe is labeled with a detectable marker. In a further embodiment, the DNA probe is labeled with a detectable marker.

15

20

This invention also provides a method of diagnosing in a subject a neoplastic condition which comprises contacting a sample from the subject with the above-described DNA probe under conditions permitting the DNA probe to hybridize with the DNA associated with the neoplastic condition, detecting the presence of hybridized DNA, and thereby diagnosing the neoplastic condition.

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This invention also provides monoclonal antibody designated Pro 1.1.; monoclonal antibody designated Pro 1.2.; monoclonal antibody designated Pro 1.3.; monoclonal antibody designated Pro 1.4.; and monoclonal antibody designated Pro 1.5.

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-30-

This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Carcinoma Tumor Antigen Gene-1. The nucleic acid molecule may be DNA, cDNA or RNA. This invention also provides isolated  
5 human nucleic acid molecule.

The nucleic acid molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and  
10 as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful  
15 methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention also provides nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing  
20 with a sequence of Prostate Carcinoma Tumor Antigen Gene-1.

This nucleic acid molecule produced can either be DNA or RNA. As used herein, the phrase "specifically  
25 hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

This nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of a Prostate Carcinoma Tumor Antigen Gene-1 can be used as a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that  
30 such probes may vary greatly in length and may be labeled  
35

-31-

with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a Prostate Carcinoma Tumor Antigen Gene-1 DNA molecule into  
5 suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be  
10 generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the Prostate Carcinoma Tumor Antigen Gene-1 DNA molecule downstream of a bacteriophage promoter such as T3, T7 or SP6. Large  
15 amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized DNA fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

20 This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Carcinoma Tumor Antigen Gene-1 operatively linked to a promoter of RNA transcription.

25 This invention also provides vectors which comprises the isolated mammalian nucleic acid molecule having the sequence of Prostate Carcinoma Tumor Antigen Gene-1. In an embodiment, the vector is a plasmid.

30 This invention also provides the plasmid designated PCTA-1. This plasmid, PCTA-1, was deposited on January 11, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the  
35 International Recognition of the Deposit of Microorganism

-32-

for the Purposes of Patent Procedure. Plasmid, PCTA-1, was accorded ATCC Accession Number 97201.

5 PCTA-1 gene contains 3850 bp. It was cloned into the XhoI and EcoRI sites of the pBluescript vector. T3 promoter is close to 5' end and T7 promoter to 3' end of PCTA-1.

10 This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA molecule encoding the Prostate Carcinoma Tumor Antigen Gene-1 protein so as to prevent translation of the mRNA molecule.

15 This invention also provides methods of detecting expression of the Prostate Carcinoma Tumor Antigen Gene-1 in a sample which contains cells comprising steps of: (a) obtaining total RNA from the cells; (b) contacting the RNA so obtained with a labelled nucleic acid molecule of  
20 claim 7 under hybridizing conditions; and (c) determining the presence of RNA hybridized to the molecule, thereby detecting the expression of the Prostate Carcinoma Tumor Antigen Gene-1 in the sample.

25 This invention provides methods of detecting expression of the Prostate Carcinoma Tumor Antigen Gene-1 in tissue sections which comprises steps of: (a) contacting the tissue sections with a labelled nucleic acid probe under hybridizing conditions permitting hybridization of the  
30 probe and the RNA of Prostate Carcinoma Tumor Antigen Gene-1; and (b) determining the presence of RNA hybridized to the probe, thereby detecting the expression of the Prostate Carcinoma Tumor Antigen Gene-1 in tissue sections.

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-33-

This invention also provides the above-described isolated mammalian nucleic acid molecule operatively linked to a promoter of RNA transcription.

5 This invention also provides a vector which comprises the above-described isolated mammalian nucleic acid molecule. In an embodiment, the vector is a plasmid. In another embodiment, the vector is a virus. In a further embodiment, the virus is a DNA virus. In a still further  
10 embodiment, the virus is an RNA virus. In another embodiment, the virus is a retrovirus.

This invention provides purified mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein. This invention  
15 also provides polypeptides encoded by the above-described isolated mammalian nucleic acid molecules.

This invention also provides antibodies capable of specifically binding to the mammalian Prostate Tumor  
20 Inducing Gene-1 protein. This invention also provides an antibody capable of competitively inhibiting the binding of the above antibodies with the mammalian Prostate Tumor Inducing Gene-1 protein. In an embodiment, these antibodies are monoclonal.

25 This invention also provides a pharmaceutical composition comprising the above-described antibody and a pharmaceutically acceptable carrier. This invention also provides a therapeutic agent comprising the above-  
30 described antibodies and a cytotoxic agent. The cytotoxic agent may be a radioisotope or toxin.

This invention provides methods for measuring the amount of a mammalian Prostate Carcinoma Tumor Antigen Gene-1  
35 protein in a biological sample comprising steps of: a)

-34-

contacting the biological sample with the specific antibody directed to the mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein under the condition permitting the formation of a complex with said antibody and the mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein, and b) measuring the amount of said complex, thereby measuring the amount of the Prostate Carcinoma Tumor Antigen Gene-1 protein in said biological sample. In an embodiment, the sample is a serum sample.

This invention also provides methods to determining whether a subject carries a cancer with metastatic potential comprising steps of: (a) measuring the amount of Prostate Carcinoma Tumor Antigen Gene-1 protein in the serum sample of the subject; and (b) comparing the amount determined in step (a) with the amount determined from the sample of a healthy subject.

This invention provides methods for determining whether a compound is capable of inhibiting the expression of Prostate Carcinoma Tumor Antigen Gene-1 protein comprising steps of: (a) contacting the transformed cells of claim 19 with an appropriate amount of the compound under conditions permitting the compound to inhibit expression of Prostate Carcinoma Tumor Antigen Gene-1 protein; and (b) detecting the level of the Prostate Carcinoma Tumor Antigen Gene-1 protein expression, a decrease in the expression level indicating that the compound is capable of inhibiting the expression of Prostate Carcinoma Tumor Antigen Gene-1 protein.

This invention provides methods for determining whether a compound is capable of specifically binding to the Prostate Carcinoma Tumor Antigen Gene-1 protein comprising steps of: (a) contacting an appropriate amount



-35-

of the purified Prostate Carcinoma Tumor Antigen Gene-1 protein with an appropriate amount of the compound under conditions permitting formation of a complex between the compound and the purified protein; (b) detecting such  
5 complex, the presence of the complex indicating that the compound is capable of binding to the receptor.

This invention provides pharmaceutical compositions comprising an effective amount of the compound capable of  
10 either binding or inhibiting the activity of the Prostate Carcinoma Tumor Antigen Gene-1 protein as determined by the above-methods and a pharmaceutical carrier.

This invention also provides a method of treating cancer  
15 with metastatic potential in a subject comprising administering effective amount of the above pharmaceutical composition, therapeutic agent alone or in combination of, to the subject.

20 This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1. The nucleic acid molecule can be DNA, cDNA, genomic DNA, synthetic DNA or RNA. This invention also provides human nucleic acid molecule.

25 This invention further provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of Prostate Tumor Inducing Gene-1.

30 This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1 operatively linked to a promoter of RNA transcription.

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-36-

This invention provides a method of detecting expression of a Prostate Tumor Inducing Gene-1 in a cell which comprises obtaining total mRNA from the cell, contacting the mRNA so obtained with a labelled nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of Prostate Tumor Inducing Gene-1 under conditions permitting hybridization, and determining the presence of mRNA hybridized to the molecule, thereby detecting the expression of the Prostate Tumor Inducing Gene-1 in the cell.

In one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules. The mRNA is then exposed to a radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

This invention also provides a method of detecting expression of a Prostate Tumor Inducing Gene-1 in tissue sections which comprises contacting the tissue sections with a labelled nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of Prostate Tumor Inducing Gene-1 under hybridizing conditions, determining the presence of mRNA hybridized to the molecule, and thereby detecting the expression of the Prostate Tumor Inducing Gene-1 in tissue sections.

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-37-

The probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues. The in-situ hybridization using a labelled nucleic acid molecule is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker and so will the expression of the Prostate Tumor Inducing Gene-1.

This invention provides an isolated mammalian nucleic acid molecule having the sequence of the Prostate Tumor Inducing Gene-1 operatively linked to a promoter of RNA transcription.

The isolated mammalian nucleic acid molecule having the sequence of the Prostate Tumor Inducing Gene-1 can be linked to vector systems. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners.

This invention also provides vectors which comprises the isolated mammalian nucleic acid molecule having the sequence of the Prostate Tumor Inducing Gene-1. In an embodiment, the vector is a plasmid.

In an embodiment, the Prostate Tumor Inducing Gene-1 sequence is cloned in EcoRI/XhoI site of the Bluescript vector. This plasmid, PTI-1, clone 18, was deposited on January 11, 1995 with the American Type Culture

-38-

Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, PTI-1, was accorded ATCC Accession Number 97020.

PTI-I gene is determined by RACE from 1 to 215 bp and the rest by PTI-1, clone. The plasmid PTI-1 is clone 18, contains 1937 bp (29 bp + 1908 bp) insert in pBluescript vector EcoRI (5' end of insert) and XhoI (3' polyA side).

The 1937 bp insert can be cut out by restriction enzymes XhoI + EcoRI. 5' end of insert is close to T3 promoter, 3' end is close to T7 promoter.

Experiments demonstrate that the 29 bp sequence of insert comes from the secondary structure of RNA so that this 29 bp sequence was not shown in complete sequence of PTI-I gene (see Fig. 8), and replaced by the right sequence obtained from RACE method.

The first 29 bp sequence is:  
5'CGGCCCCGAGCTCGTGCCGAATTCGGCCCCGAGAGCGTTAAAGTGTGATGGCGTACATCTT. The sequence from 30-1937 bp (1907 bp) is the sequence 216-2,123 bp (1907 bp) in complete sequence of PTI-1 (Fig. 8).

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which hybridize with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which

-39-

cuts at that site. Other means to obtain the vectors are also available and known to an ordinary skilled practitioner.

5 This invention provides a host vector system for the production of a polypeptide having the biological activity of a mammalian Prostate Tumor Inducing Gene-1 protein which comprises the above described vectors and a suitable host. These vectors may be transformed into  
10 a suitable host cell to form a host cell vector system for the production of a polypeptide.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and  
15 transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector  
20 includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by  
25 methods well known in the art, for example, the methods described above for constructing vectors in general.

This invention also provides a method of producing a polypeptide having the biological activity of a mammalian  
30 Prostate Tumor Inducing Gene-1 protein which comprises growing the host cells of the above described host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

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-40-

This invention also provides mammalian cells comprising the above-described nucleic acid molecule.

5 This invention provides purified mammalian Prostate Tumor Inducing Gene-1 protein. This invention also provides a polypeptide encoded by the isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1 protein.

10 This invention also provides a method to produce an antibody using the above-described mammalian Prostate Tumor Inducing Gene-1 protein.

15 This invention provides an antibody capable of binding specifically to the mammalian Prostate Tumor Inducing Gene-1 protein. In an embodiment, the antibody is a monoclonal antibody.

20 This invention also provides a therapeutic agent comprising the above-described antibody and a cytotoxic agent. In an embodiment, the cytotoxic agent is either a radioisotope or toxin.

25 This invention further provides an immunoassay for measuring the amount of a mammalian Prostate Tumor Inducing Gene-1 protein in a biological sample comprising steps of: a) contacting the biological sample with at least one of the above-described antibodies to form a complex with said antibody and the mammalian Prostate  
30 Tumor Inducing Gene-1 protein; and b) measuring the amount of the Prostate Tumor Inducing Gene-1 protein in said biological sample by measuring the amount of said complex.

35 This invention also provides a method of inactivating

-41-

oncogenic transformation of cells comprising inactivating the expression of the expression of the 5'-UTR of Prostate Tumor Inducing Gene-1. In an embodiment, the the inactivation is carried out by deleting the complete  
5 or a portion of the 5'-UTR sequence.

This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-2. The nucleic acid molecule can be DNA,  
10 cDNA, genomic DNA, synthetic DNA or RNA. This invention also provides human nucleic acid molecule.

This invention further provides a nucleic acid molecule of at least 15 nucleotides capable of specifically  
15 hybridizing with a sequence of Prostate Tumor Inducing Gene-2.

This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor  
20 Inducing Gene-2 operatively linked to a promoter of RNA transcription.

This invention also provides vectors which comprises the isolated mammalian nucleic acid molecule having the  
25 sequence of Prostate Tumor Inducing Gene-2. In an embodiment, the vector is a plasmid.

This invention also provides the plasmid designated PTI-2. This plasmid, PTI-2, was deposited on January 11,  
30 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The plasmid, PTI-  
35 2, was accorded with ATCC Accession Number 69742.

-42-

PTI-2 contains 1819 bp DNA. It was cloned into the XhoI and EcoRI site of the pBluescript vector. T3 promoter is close to the 5'-end and T7 promoter to the 3'-end of PTI-2. The (1819 bp) insert of PTI-2 can be cut out with XhoI and EcoRI enzyme.

This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-3. The nucleic acid molecule can be DNA, cDNA, genomic DNA, synthetic DNA or RNA. This invention also provides human nucleic acid molecule.

This invention further provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of Prostate Tumor Inducing Gene-3.

This invention provides an isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-3 operatively linked to a promoter of RNA transcription.

This invention also provides vectors which comprises the isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-3. In an embodiment, the vector is a plasmid.

This invention also provides the plasmid designated PTI-3. This plasmid, PTI-3, was deposited on January 11, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, PTI-3, was accorded ATCC Accession Number 97022.



-43-

PTI-3 is a partial sequence of the gene. A 1869 bp DNA of PTI-3 is inserted into PCR<sup>TM</sup>II vector (3.9 kb). 5'-end of the insert is adjacent to Sp-6 promoter and 3'-end is adjacent to T7 promoter.

5

The insert can be cut out with EcoRI restriction enzyme to obtain the 1869 bp DNA (with extra 5 bp vector sequence at both sides).

10 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which  
15 follow thereafter.

-44-

**First Series of Experiments**  
**Materials and Methods**

**Cell types and Culture Conditions**

5

The CREF-Trans 6 cells line (11) is a specific subclone of the Fischer F2408 rat embryo fibroblast (CREF) cell line that displays an increased sensitivity for expression of transfected genes compared with parental CREF cells (12). The LNCaP cell line was derived from metastases from a patient with advanced prostate cancer (13) and was provided by Dr. A. K. Ng (Department of Pathology, Columbia University, College of Physicians and Surgeons, New York, N.Y.) and Dr. Steven Harris (W. Alton Jones Cell Science Center, New York, N.Y.). CREF-Trans 6:4 NMT cells were derived from a tumor induced in a nude mouse following injection of G418-resistant CREF-Trans 6 cells cotransfected with DNA from LNCaP cells and a dominant-acting neomycin resistance gene (pSV2neo) (11). CREF-Trans 6:4-7 NMT cells were derived from a tumor induced in a nude mouse following injection of G418-resistant CREF-Trans 6 cells cotransfected with DNA from CREF-Trans 6:4 NMT cells and the pSV2neo plasmid (11). MDR CREF-Trans 6 cells were produced by transfecting CREF-Trans 6 cells with an expression vector plasmid containing a human MDR (also known as PGY1) gene (pHaMDR1/A) (14), which was provided by Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD.) and selecting for colchicine resistance as previously described (15).

For this study, four independent CREF-Trans 6 MDR clones were used: 1) CREF-Trans 6:MDR A1, 2) CREF-Trans 6:MDR C3, 3) CREF-Trans 6:MDR D2, and 4) CREF-Trans 6:MDR F4. All four CREF-Trans 6 MDR clones displayed increased

-45-

resistance to colchicine versus parental CREF-Trans 6 cells, and they were cross-resistant to vincristine, doxorubicin, and dactinomycin (data not shown). The human prostatic carcinoma cell lines DU-145 and PC-3 were  
5 obtained from the American Type Culture Collection (Rockville, MD.) The human breast carcinoma cell line MCF7 was provided by Dr. John W. Greiner (National Cancer Institute). MCF7 CL4 C1 (MCF7 CL4) is a single-cell-  
10 derived subclone of MCF7 established in one of applicants' laboratories at Columbia University. MDR MCF7 CL4 subclones (MCF7 CL4:MDR I, MCF7 CL4:MDR II and MCF7 CL4:MDR III) were obtained in a manner similar to that used for CREF-Trans 6 MDR clones. MCF7 CL4:MDR I,  
15 MCF7 CL4:MDR II and MCF7 CL4:MDR III cells contained MDR1 messenger RNA (mRNA), expressed the 170-kd P-glycoprotein, and displayed increased resistance to colchicine and vincristine compared with MCF7 and MCF7 CL4 cells (data not shown). GBM-18 tumor cells were  
20 derived from a patient with a stage IV astrocytoma (glioblastoma multiforme) (16). Normal human skin fibroblasts, NHSF-1, were established from a skin biopsy and provided by Dr. Armand F. Miranda (Department of Pathology, Columbia University) (17). The human melanoma cell line H0-1 was provided from a 49-year-old woman and  
25 was provided by Dr. Beppino Giovanella (Stehlin Foundation for Cancer Research, Houston, Tex.) (18). The human melanoma cell line MeWo was provided by Dr. Robert S. Kerbel (Sunnybrook Health Science Center, Toronto, Canada). The human colon carcinoma cell lines WiDr and  
30 LS174T were provided by Dr. John W. Greiner.

CREF-Trans 6, CREF-Trans 6 MDR subclone, CREF-Trans 6:4 NMT, CREF-Trans 6:4-7 NMT, H0-1 and MeWo cells were grown, at 37°C in Dulbecco's modified Eagle's medium  
35 supplemented with 5% fetal bovine serum. Normal human

-46-

5 skin fibroblasts (NHSF-1) as well as the LNCaP ,WiDr, LS174T, MCF7 and MCF7 CL4 cells and the MCF7 CL4 MDR subclone cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cells were maintained in the logarithmic phase of growth by culturing at a 1:5 or 1:10 ratio of resuspended cells to fresh medium prior to confluence.

10 **Preparation of Mouse Polyclonal Antibodies,  
Enzyme-Linked Immunosorbent Assay, and SEM**

15 BALB/c female mice (8-10 weeks old) (Charles River Breeding Laboratories, Wilmington, Mass.) were hyperimmunized with CREF-Trans 6 cells. Their care was in accordance with institutional guidelines. Animals received one subcutaneous injection of manually resuspended CREF-Trans 6 cells mixed with complete Freund's adjuvant (1:1) on day 0. On day 7, animals received a subcutaneous injection of manually resuspended CREF-Trans 6 cells mixed with incomplete Freund's adjuvant (1:1). On days 14 and 21, animals received an intraperitoneal injection of manually resuspended CREF-trans 6 cells in Hanks' phosphate-buffered solution. On day 35, mice were bled from the retro-orbital eye socket, and the sera were prepared and tested for anti-CREF-Trans 6 activity by enzyme-linked immunosorbent assay. For these assays, CREF-Trans 6 cells were grown in 96-well microtiter plates to near confluence. Cells were fixed with 3.7% formalin in phosphate-buffered solution (5 minutes at room temperature) and blocked with 10% normal goat serum (60 minutes at 37°C ). The anti-CREF-Trans 6 antisera were titered against fixed CREF-Trans 6 cells (serial dilutions of antisera, 2 hours at 37°C). Binding to CREF-Trans 6 cells was detected using a goat anti-mouse immunoglobulin secondary antibody conjugated to

-47-

horseradish peroxidase (60 minutes at 37°C). 3,3', 5,5'-tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, M.D.) was added in the presence of H<sub>2</sub>O<sub>2</sub> and positive binding was monitored by a color change and quantitated by spectrophotometer (19).

In this study, the SEM procedure involved the coating of transfected CREF-Trans 6 cells with high-titer mouse anti-CREF-Trans 6 antisera to block the rat antigenic molecules prior to hyperimmunizing BALB/c mice. One to 3 million transfected CREF-Trans 6 cells were incubated overnight at 4°C with a 1:100 dilution of mouse anti-CREF-Trans 6 antisera. Prior to the injection of polyclonal antibody-coated transfected CREF-Trans 6 cells into BALB/c mice, cells were first incubated in 1% neutral-buffered formalin for 5 minutes at 4°C. Mice were given four injections of formalin-fixed cells over a 21-day period using a protocol similar to that utilized for developing mouse anti-CREF-Trans 6 antisera. The spleens of hyperimmunized mice were removed, and spleen cells were isolated and fused with NS1 murine myeloma cells (American Type Culture Collection) to form hybridomas as previously described (19).

## 25 Immunoprecipitation Analysis

Immunoprecipitation analysis was performed as described previously (20). CREF-Trans 6, CREF-Trans 6:4 NMT, CREF-Trans 6:4-7 NMT, LNCaP, and DU-145 cells were grown to 80% confluence in 6-cm plates, starved of methionine for 1 hour at 37°C in methionine-free medium (20) and labeled for 2 hours at 37°C in 1 mL of the same medium with 1 mCi of [<sup>35</sup>S] methionine (Express <sup>35</sup>S; NEN Chemicals, Boston, Mass). Cell lysates were prepared and immunoprecipitated with the Pro-1.4 monoclonal antibody (produced by

-48-

hybridomas prepared using the SEM procedure with CREF-Trans 6:4 NMT cells) as described previously (20).

#### Fluorescence-Activated Cell Sorter Analysis

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Fluorescence-activated cell sorter (FACS) analysis was performed as described previously (21,22). Results are expressed as mean fluorescence intensity units. Monoclonal antibodies specific for human leukocyte antigen class I antigens were supplied by Dr. Soldano Ferrone (New York Medical College, Valhalla). All studies were performed a minimum of three times with duplicate samples in each experiment. Replicate samples within individual experiments varied 10% or less, and the variation between experiments was generally 20% or less.

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#### Experimental Results

##### Development of Monoclonal Antibodies Reacting With MDR CREF-Trans 6 and MCF7 cells

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To determine the feasibility of the SEM approach for developing monoclonal antibodies reactive with cell-surface antigens on transfected target cells, applicants performed initial studies using a defined molecule expressed on the cell surface, i.e., the typical MDR gene product. A schematic of the SEM protocol is shown in Fig. 1. Overexpression of the MDR1 gene results in an increased quantity of the 170-kd membrane glycoprotein (P-glycoprotein), which functions as an adenosine triphosphate-dependent drug efflux pump [reviewed in(23)]. CREF-Trans 6 cells were transfected with the pHaMDR1/A expression vector (14), and clones surviving in colchicine were isolated. CREF-Trans 6:MDR clones produced MDR1 mRNA, expressed the 170-kd P-glycoprotein,

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-49-

and displayed cross-resistance to other chemotherapeutic agents, including vincristine, doxorubicin, and dactinomycin (data not shown). An MDR CREF-Trans 6 clone (i.e., CREF-Trans 6:MDR A1) was used in combination with the SEM procedure to generate monoclonal antibodies specific for the MDR P-glycoprotein. CREF-Trans 6:MDR A1 cells were coated with polyclonal antibody produced against CREF-Trans 6 cells fixed in formalin and injected four times over a 21-day period into BALB/c mice. Spleen cells were isolated and fused with the NS1 murine myeloma cell line, resulting in hybridomas secreting monoclonal antibodies reacting with outer epitopes of the P-glycoprotein on additional independently derived CREF-Trans 6:MDR clones (Fig. 2). The four monoclonal antibodies specific for the P-glycoprotein, MDR 2.3, MDR 3.6, MDR 8.12 and MDR 9.7, reacted with CREF-Trans 6:MDR A1, CREF-Trans 6:MDR C3, CREF-Trans 6:MDR D2, and CREF-Trans 6:MDR F4 cells. In contrast, the different SEM-derived MDR monoclonal antibodies did not react with a large number of non-MDR cells, including CREF-Trans 6, CREF-Trans 6:4 NMT, LNCaP, MCF7, WiDr, LS174T, H0-1, MeWo, NHTSF-1 or GBM-18 (data not shown).

Applicants then determined if an additional cell type expressing the same MDR1 gene and the MDR phenotype as CREF-Trans 6:MDR A1 cells also contained the same P-glycoprotein surface antigenic epitopes. MDR MCF7 CL4 cells were developed by transfection with pHaMDR1/A and selection for colchicine resistance. MCF7 parental cells and the single-cell-derived MCF7 subclone, MCF7 CL4, did not display the MDR phenotype and did not react with monoclonal antibodies MDR 2.3, MDR 3.6, MDR 8.12, or MDR 9.7 (Fig 3). However, both MCF7 and MCF7 CL4 cells reacted with human leukocyte antigen class I monoclonal antibodies. A series of independently derived MDR MCF7

-50-

CL4 subclones, including MCF7 CL4:MDR I, MCF7 CL4:MDR II and MCF7 CL4:MDR III cells, was found to react with both the human leukocyte antigen class I and SEM-derived MDR monoclonal antibodies (Fig. 3). These results indicate that monoclonal antibodies developed using the SEM approach with transfected CREF-Trans 6 cells can also react with additional cell types expressing the same surface-localized molecules.

#### 10      Development of Monoclonal Antibodies Reacting With Human Prostatic Carcinoma Cells

Cotransfection of CREF-Trans 6 cells with high-molecular-weight DNA from the human prostatic carcinoma cell line LNCaP and pSV2neo plasmid, followed by selection for G418 resistance and injection into nude mice, results in tumor formation (11). To determine if tumor-derived CREF-Trans 6 cells display novel surface molecules related to the original transforming human tumor DNA, applicants used the SEM procedure with a primary nude mouse tumor-derived cell line, CREF-Trans 6:4 NMT (11). Cells were coated with CREF-Trans 6 polyclonal antibodies, fixed in formalin, and injected repeatedly into BALB/c mice. As described above for MDR CREF-Trans 6 cells, hybridomas were produced, and specific hybridomas were identified that produced monoclonal antibodies reacting with both primary tumor-derived (CREF-Trans 6:4 NMT) and secondary tumor-derived (CREF-Trans 6:4-7 NMT) cells (Fig 4). These monoclonal antibodies, designated Pro 1.1, Pro 1.2, Pro 1.3, Pro 1.4 and Pro 1.5 did not react by FACS analysis with CREF-Trans 6, NHSF-1, GBM-18, WiDr, LS174T, MeWo, or H0-1 cells (data not shown). All five monoclonal antibodies did, however, react with LNCaP cells, and specific Pro monoclonal antibodies also reacted (as demonstrated by FACS analysis) with two



-51-

additional human prostatic carcinoma cell lines, PC-3 and DU-145.

5 The degree of surface binding of the different Pro monoclonal antibodies to the same cell type varied, suggesting that these monoclonal antibodies may recognize different epitopes on the same tumor-associated antigen. With the majority of the Pro monoclonal antibodies, binding was greater with LNCaP cells than with CREF-Trans  
10 6:4 NMT or CREF-Trans 6:4-7 NMT cells. In the case of PC-3 and DU-145 human prostatic carcinoma cells, four (1.2, 1.3, 1.4 and 1.5) of the five Pro monoclonal antibodies bound to PC-3 cells, whereas low-level binding was apparent only with Pro 1.2, 1.4 and 1.5 in DU-145  
15 cells. Preliminary FACS analysis also indicated that Pro 1.1, 1.3 and 1.5 displayed significant binding to the surface of two human breast carcinoma cell lines, T47D and MCF7 (data not shown).

20 To obtain additional information about the Pro monoclonal antibodies generated using the SEM approach, applicants performed immunoprecipitation analysis of polypeptides encoded by transfected CREF-Trans 6 and human prostatic carcinoma cells (Fig. 5). Cells were labeled with [<sup>35</sup>S]  
25 methionine, and cell lysates were prepared and combined with monoclonal antibody Pro-1.4. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). A protein of approximately 42 kd was immunoprecipitated from cell lysates produced from  
30 CREF-Trans 6:4 NMT, CREF-Trans 6:4-7 NMT, LNCaP, and DU-145 (Fig. 5). In contrast, this potentially new tumor-associated antigen was not detected in cell lysates obtained from CREF-Trans 6, NHSF-1, GBM-18, WiDR, MeWo, or H0-1 cells (Fig. 5 and data not shown). The relative  
35 quantity of the immunoprecipitated 42 kd tumor-associated

-52-

antigen was greatest in CREF Trans 6:4-7 NMT and LNCaP cells which also displayed the highest level of surface binding using monoclonal antibody Pro 1.4 and FACS analysis (Fig 4). These results indicate that the SEM procedure can be used to generate monoclonal antibodies recognizing surface-expressed human antigens expressed on transfected CREF-Trans 6 cells containing unidentified human transforming genes that encode tumor-associated antigens expressed on the cell surface.

### Experimental Discussion

In many classes of neoplastic cells, unique sets of tumor-associated antigens are present that are not expressed or are expressed at lower levels compared with normal cellular counterparts [reviewed in (2-6)]. The classical approach for detecting these molecules is to use intact cells or cell membrane preparations from tumor cell lines or primary tumor samples to generate hybridomas, producing monoclonal antibodies reacting with specific tumor-associated antigens [reviewed in (1-3, 6)]. This approach is laborious and often unsuccessful in generating monoclonal antibodies that display the necessary specificity to permit their use for cancer diagnostics or therapeutics (3,4,6). An alternative strategy, which is dependent upon a functional change induced in a target cell, involves the use of DNA transfection and an approach termed "surface-epitope masking." In the present study, applicants demonstrate the utility of this combined strategy for the generation of monoclonal antibodies specific for a known surface expressed molecule, the P-glycoprotein-mediating MDR, and the product of an unidentified putative human prostatic carcinoma gene. The SEM approach has also been used to produce monoclonal antibodies reacting with the human  $\gamma$ -

-53-

interferon receptor (Su, Z-z., Pestka, S., Fisher, P.B.: manuscript in preparation) and an unidentified putative human breast carcinoma gene (Yemul, S., Su, Z-z., Leon, J.A., et al: manuscript in preparation) expressed in  
5 CREF-Trans 6 cells. All of these results indicate that the combination of transfection and SEM offers a unique opportunity to generate monoclonal antibodies specific for human tumor-associated antigens without prior knowledge of the identity of the gene encoding these  
10 products. With appropriate expression vector gene constructs, this strategy can also be used to generate monoclonal antibodies reacting with well-characterized, surface-localized proteins expressed in CREF-Trans 6 cells. This combined approach, at least in principle,  
15 should be applicable to any experimental model in which specific changes occur in the expression of surface molecules between a "tester" (a transfected cell expressing a new surface-expressed molecule) and a "driver" (the untransfected parental cell).

20 The identity and function of the putative tumor-inducing human prostatic carcinoma gene that has been stably transferred from LNCaP cells to CREF-Trans 6 cells are not known. However, CREF-Trans 6:4 NMT cells that  
25 contain this potential prostatic carcinoma gene can be used to generate monoclonal antibodies reactive with surface antigens on both LNCaP-transfected cells and human prostatic carcinoma cell lines. This ability suggest a potential causal relationship between  
30 expression of the transfected gene and expression of the prostatic carcinoma phenotype.

Prior studies (24,25) have indicated that transformed NIH 3T3 cells transfected with specific human tumor DNAs  
35 could be used to generate monoclonal antibodies specific

-54-

for surface antigens expressed by the original tumor cell line as well as histologically similar tissue types. This approach has been used to generate monoclonal antibodies specific for cell-surface antigens expressed on NIH 3T3 cells transformed by human pancreatic carcinoma (24) and acute myelogenous leukemia (25) DNA. The monoclonal antibodies produced against DNA-transformed NIH 3T3 cells from human pancreatic carcinoma reacted with surface antigens on transfected transformed cells, the original human pancreatic carcinoma cell line, and six additional human pancreatic carcinoma cell lines (24). These monoclonal antibodies did not react with untransfected NIH 3T3 cells or human lymphoblastoid, melanoma, prostatic carcinoma, or normal human skin fibroblast cell lines (24). Results from both studies (24, 25) indicate that the combination of DNA transfection and monoclonal antibody development may prove useful in generating monoclonal antibodies with specificity for cell-surface epitopes displayed by different histologic tumor types. In this respect, the ability of CREF-Trans 6 to identify tumor-inducing genes without biological activity in NIH 3T3 cells indicates that this new human tumor DNA transfection-acceptor cell line may prove useful for the identification and cloning of potentially novel genetic elements mediating specific human cancers.

In the present study, the SEM approach was used to stimulate the production of spleen cells reactive with cell-surface-accessible molecules. Spleen cells were then used to produce hybridomas that secrete monoclonal antibodies reactive with accessible surface antigens. The SEM approach described in this manuscript uses a formalin fixation step prior to immunizing animals with polyclonal antibody-coated tester cells. This procedure

-55-

was originally adopted to more efficiently produce monoclonal antibodies that would have direct diagnostic potential, i.e., they could be used to detect antigens on formalin-fixed tissue. Monoclonal antibodies produced using the SEM procedure have demonstrated specificity for viable cells, frozen tissue specimens, and both formalin-fixed tissue specimens and formalin-fixed cells. The SEM procedure described in this application would not be predicted to generate monoclonal antibodies reactive with fixative-sensitive antigens. However, modifications of the SEM approach using procedures other than formalin fixation, including injection of unfixed antibody-coated cells or use of novel immune complexes (26), should result in the generation of monoclonal antibodies reacting with fixative sensitive antigenic epitopes of molecules expressed on the cell surface.

Recently, an approach called "phage display combinatorial libraries" has been developed in which combinatorial complementary DNA (cDNA) libraries are prepared in phage directly from antigen-stimulated spleen cells (27,28). In this context, transfected cells that had been subject to the SEM procedure could be utilized as immunogens to stimulate an antigenic response that could then be followed by the isolation of spleen cells and generation of combinatorial phage cDNA libraries. This approach could then result in the direct identification of potentially critical genes involved with transformation and with genes that encode specific human tumor-associated antigens and other molecules expressed on the cell surface.

The SEM approach has been performed using murine monoclonal antibodies to coat rat antigenic surface epitopes on a rat embryo fibroblast cell line, CREF-Trans

-56-

6. Alternatively, it should be possible to use transfected CREF-Trans 6 cells as direct immunogens in syngeneic Fischer rats for the generation of rat hybridomas or rat x mouse heteromyelomas. Although these studies are still in progress, it is apparent that the SEM approach employing murine monoclonal antibody-coated CREF-Trans 6 is preferable to injection of transfected CREF-Trans 6 cells directly into syngeneic rats. Murine monoclonal antibodies are relatively easy to produce and are highly amenable to purification in large quantities. In addition, the technologies required for the genetic manipulation of murine monoclonal antibodies (e.g., chimerization, humanization, and bispecific monoclonal antibodies) are readily available (29-31). These genetic approaches are extremely important if a monoclonal antibody is to be used in human clinical trials for imaging or as a therapeutic agent (4-6).

The theoretical basis of the SEM approach involves antigenic subtraction, i.e., the blocking of antigenic sites shared by two genetically similar cell types. This process results in an increase in the sensitivity of detection of novel surface antigens. The present studies have emphasized applications of the SEM approach, using transfected cells expressing known as well as unidentified cell-surface molecules. However, many additional situations that would be adaptable to the SEM procedure can be envisioned. For example, the SEM protocol could be used to develop monoclonal antibodies specific for surface changes occurring in metastatic tumor cells. To achieve this goal, polyclonal antibodies could be generated against a primary tumor, and these polyclonal antibodies could be used to mask surface epitopes on metastatic tumors. This step would be performed prior to sensitizing animals for the

-57-

development of hybridomas or combinatorial phage cDNA expression libraries specific for surface-expressed metastatic antigens. Similarly, polyclonal antibodies could be generated against normal tissue of a specific histologic type, and these polyclonal antibodies could then be used to mask surface epitopes on a histologically similar tumor derived from the same patient. The cells with masked surface epitopes could then be injected into animals so that they would develop sensitized spleen cells for the development of hybridomas or combinatorial phage cDNA libraries specific for tumor-associated antigens. SEM would also appear to be ideally suited for the development of monoclonal antibodies specific for the outer domain of membrane-localized growth factor receptors and cell-membrane transporter proteins. Future applications of the SEM approach could also result in the development of monoclonal antibodies and/or the isolation of relevant genes involved in determining tumor cell recognition by both nonspecific and specific immunologic effector cells, mediating atypical multidrug resistance, and identifying mediators of autoimmune diseases.

-58-

References of the First Series of Experiments

1. Goding, J.W., (1980) Antibody production by hybridomas, J. Immunol. Methods, 39:285-308.
2. Schlom, J., Colcher, D., Hand, P.H., et al. (1985) Monoclonal antibodies reactive with breast tumor-associated antigens, Adv. Cancer Res. 43:143-173.
3. Epstein, A.L., Khawli, L.A., (1991) Tumor biology and monoclonal antibodies: overview of basic principles and clinical considerations, Antibody Immunoconjugates Radiopharmaceuticals, 4:373-384.
4. Waldmann, T.A., (1991) Monoclonal antibodies in diagnosis and therapy, Science, 252:1657-1662.
5. Pirofski, L.A., Casadevall, A., Scharff, M.D., (1992) Current state of hybridoma technology, Am. Soc. Microbiol. News, 58:613-617.
6. Leon, J.A., Goldstein, N.I., Fisher, P.B., New approaches for the development and application of monoclonal antibodies for the diagnosis and therapy of human cancer. Pharmacol Therapeut., in press.
7. Varmus, H., (1984) The molecular genetics of cellular oncogenes, Annu. Rev. Genet., 18:533-612.
8. Weinberg, R.A., (1984) The action of oncogenes in the cytoplasm and the nucleus, Science, 230:770-776.
9. Bishop, M.J., (1987) The molecular genetics of cancer, Science, 235:305-311.



-59-

10. Barbacid, M., (1987) Ras genes, Annu. Rev. Biochem., 56:779-827.
- 5 11. Su, Z-z., Olsson, C.A., Zimmer, S.G., et al. (1992) Transfer of a dominant-acting tumor-inducing oncogene from human prostatic carcinoma cells to cloned rat embryo fibroblast cells by DNA-transfection, Anticancer Res. 12:297-304.
- 10 12. Fisher, P.B., Babiss, L.E., Weinstein, I.B., et al. (1982) Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF), Proc. Natl. Acad. Sci., USA, 79:3527-3531.
- 15 13. Horoszewicz, J.S., Leong, S.S., Kawinski, E., et al. (1983) LNCaP model of human prostatic carcinoma, Cancer Res., 43:1809-1818.
- 20 14. Kane, S.E., Troen, B.R., Gal, S., et al. (1988) Use of a cloned multidrug resistance gene for coamplification and overproduction of major excreted protein, a transformation-regulated secreted acid protease, Mol. Cell Biol., 8:3316-3321.
- 25 15. Reddy, P.G., Graham, G.M., Datta, S., et al. (1991) Effect of recombinant fibroblast interferon and recombinant immune interferon on growth and the antigenic phenotype of multidrug-resistant human glioblastoma multiforme cells, J. Natl. Cancer Inst. 83:1307-1315.
- 30 16. Vita, J.R., Edwals, G.M., Gorey, T., et al. (1988) Enhanced in vitro growth suppression of human glioblastoma cultures treated with the combination of recombinant fibroblast and immune interferons,
- 35

-60-

Anticancer Res., 8:297-302.

- 5 17. Su, Z-z., Grunberger, D., Fisher, P.B., (1991) Suppression of adenovirus type 5 E1A-mediated transformation and expression of the transformed phenotype by caffeic acid phenethyl ester (CAPE), Mol. Carcinog., 4:231-242.
- 10 18. Giovanella, B.C., Stehlin, J.S., Jr., Santamaria, C., et al. (1976) Human neoplastic and normal cells in tissue culture: I. Cell lines derived from malignant melanomas and normal melanocytes, J. Natl. Cancer Inst., 56:1131-1142.
- 15 19. Goldstein, N.L., Nagle, R., Villar, H., et al. (1990) Isolation and characterization of a human monoclonal antibody which reacts with breast and colorectal carcinoma, Anticancer Res., 10:1491-1500.
- 20 20. Duigou, G.J., Su, Z-z., Babiss, L.E., et al. (1991) Analysis of viral and cellular gene expression during progression and suppression of the transformed phenotype in type 5 adenovirus-transformed rat embryo cells, Oncogene, 6:1813-1824.
- 25 21. Guarini, L., Temponi, M., Edwalds, G.M., et al. (1989) In vitro differentiation and antigenic changes in human melanoma cell lines, Cancer Immunol. Immunother., 30:262-268.
- 30 22. Leon, J.A., Mesa-Tejada, R., Gutierrez, M.C., et al. (1989) Increased surface expression and shedding of tumor associated antigens by human breast carcinoma cells treated with recombinant human interferons or phorbol ester tumor promoters, Anticancer Res., 35

-61-

9:1639-1647.

23. Gottesman, M.M., Pastan, I., (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter; Annu. Rev. Biochem., 62:385-427.  
5
24. Hollingsworth, M.A., Rebellato, L.M., Moore, J.W.. et al. (1986) Antigens expressed on NIH 3T3 cells following transformation with DNA from a human pancreatic tumor, Cancer Res., 46:2482-2487.  
10
25. Scuderi, P., Westin, E., Clagett, J., et al. (1985) Detection of surface antigen in NIH 3T3 cells transfected with a human leukemia oncogene, Med. Oncol. Tumor Pharmacother., 2:233-242.  
15
26. Songsakphisarn, R., Goldstein, N.I., (1993) The use of a novel immune complex to isolate neutralizing antibodies to basic fibroblast growth factor, Hybridoma, 12:343-348.  
20
27. Marks, J.D., Hoogenboom, H.K., Bonnert, T.P., et al. (1991) By-passing immunization: human antibodies from V-gene libraries displayed on phage, J. Mol. Biol., 222:581-597.  
25
28. Huse, W.D., Sastry, L., Iverson, S.A., et al. (1989) Generation of a large combinational library of the immunoglobulin repertoire in phage lambda, Science, 249:1275-1281.  
30
29. Tan, L.K., Or, V.T., Morrison, S.L., (1985) A human-mouse chimeric immunoglobulin gene with a human variable region is expressed in mouse myeloma cells, J. Immunol., 135:3564-3567.  
35

-62-

30. Co, M.S., Deschamps, M., Whitley, R.J., et al. (1991) Humanized antibodies for antiviral therapy, Proc. Natl. Acad. Sci, USA, 88:2869-2873.
- 5 31. Staerz, U.D., Bevan, M.J., (1986) Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity, Proc. Natl. Acad. Sci, USA, 83:1453-1457..

-63-

**Second Series of Experiments**

Elucidating the relevant genomic changes mediating development and evolution of prostate cancer is paramount for effective diagnosis and therapy. Using an improved DNA-acceptor cell line, CREF-Trans 6, and cotransfection techniques, with human prostatic carcinoma DNA, a putative dominant-acting nude mouse tumor-inducing oncogene, PTI-1, has been identified and cloned.

Differential RNA display reveals a novel 214 bp DNA fragment representing a differentially expressed RNA in tumor-derived transfected cells. Screening of a human prostatic carcinoma (LNCaP) cDNA library with the novel 214 bp DNA sequence identifies a full-length 2.0 Kb PTI-1 cDNA. Sequence analysis indicates that PTI-1 is a novel gene containing a unique 630 bp 5' sequence and a 3' sequence homologous to a truncated and mutated form of human elongation factor-1 alpha. In vitro translation demonstrate that the PTI-1 cDNA encodes a predominant ~46 kDa protein. Probing Northern blots with a DNA fragment corresponding to the 5' region of the PTI-1 gene identifies multiple PTI-1 transcripts in RNAs from LNCaP-transfected tumor-derived CREF-Trans 6 cells and human carcinoma cell lines derived from the prostate, lung, breast and colon. In contrast, PTI-1 RNA is not present in human melanoma, neuroblastoma, osteosarcoma, normal cerebellum or glioblastoma multiforme cell lines. Using a pair of primers recognizing a 279 bp region within the unique 630 bp 5' PTI-1 sequence, RT-PCR detects PTI-1 expression in patient-derived prostate carcinomas, but not in normal prostate or benign prostatic hypertrophy (BPH). In contrast, RT-PCR detects prostate-specific antigen (PSA) expression in all prostates tissue specimens. These results indicate that PTI-1 is a novel putative oncogene that may contribute to carcinoma

-64-

development in human prostate and other tissues. The approaches used, rapid expression cloning with the CREF-Trans 6 system and the differential RNA display strategy, should prove widely applicable for identifying and cloning additional novel human oncogenes.

The American Cancer Society estimates that 200,000 American men will have been diagnosed with prostate cancer in 1994 and 38,000 afflicted men will have died of this disease (1). The current methods for detecting early prostate cancer are limited in both their sensitivity and specificity (2). These include physical examination that might easily miss small or centrally located tumors, serum prostate-specific antigen (PSA) determination that is not specific to malignant prostate disease, and tissue biopsy in which sampling error may lead to erroneous benign diagnosis (3,4). Predictors and early detection of therapeutic relapse such as monitoring of PSA levels, ultrasound and bone scans are also unsatisfactory, as these require fairly bulky tumor regrowth before discovery (5,6). Using current approaches a high percentage, 40 to 50%, of patients considered to have clinically localized disease actually contain understaged diseases subsequent to radical surgery (7,8). Surgical intervention is not considered the appropriate treatment protocol for patients with progressive disease. These findings emphasize the need for improved diagnostic and therapeutic approaches for identifying prostate carcinomas and for predicting clinical aggressiveness.

A primary objective of investigators studying cancer etiology is the identification of gene(s) within tumor cells with oncogenic potential. A procedure to achieve this goal involves the transfer of high molecular weight (HMW) DNA from established tumor cell lines or primary

-65-

tumors into appropriate acceptor cell lines by DNA-transfection (9). Target cells are then examined for morphological transformation, i.e., focus formation. A modification of this approach involves cotransfection of target cells with HMW DNA plus a selectable antibiotic resistance gene, such as pSV2neo, selection for antibiotic resistance and then injection of pooled antibiotic resistant cells into nude mice to identify clones of cells with tumorigenic potential (10). The majority of studies using these approaches have relied on the immortal murine cell line NIH-3T3 (9,10). Unfortunately, NIH-3T3 cells generally prove unsuccessful in identifying novel dominant-acting oncogenes from human tumor cell lines or clinical samples and even when successful, subsequent cloning indicates genetic elements not relevant to the majority of human cancers. These studies accentuate the need for better techniques to identify dominant-acting human cancer genes and for more suitable target cell lines to detect novel tumor-inducing oncogenes.

Recent studies using the cotransfection/nude mouse tumor assay with HMW DNA from a human prostatic carcinoma cell line, LNCaP (11), and a new DNA-acceptor cell line, CREF-Trans 6 (12), indicate the presence of a dominant-acting tumor-inducing gene (12). Applicants have presently cloned and characterized a novel gene, prostate carcinoma tumor inducing gene 1 (PTI-1), using the differential RNA display (DD) technology (13), library screening strategies (14,15) and the RACE procedure (14). The full-length PTI-1 cDNA consists of 2,123 nucleotides and contains a novel 630 nt region sharing sequence homology with bacterial ribosomal 23S RNA fused to a sequence that is a truncated and mutated form of human elongation factor-1 alpha (EF-1 $\alpha$ ). LNCaP-transfected tumor-derived

-66-

5 CREF-Trans 6 cells as well as human prostate carcinoma  
cell lines and patient-derived prostate carcinomas  
express PTI-1. In contrast, PTI-1 RNA is not evident  
using RT-PCR in normal prostate or benign prostatic  
hypertrophy (BPH) tissue samples. PTI-1 expression occurs  
10 in additional human carcinomas, including breast, colon  
and lung, but not in normal cerebellum, glioblastoma  
multiforme, melanoma, neuroblastoma or osteosarcoma cell  
lines. These observations indicate that PTI-1 is a novel  
genetic element displaying expression in specific human  
15 carcinomas and implicates mutagenic changes in EF-1 $\alpha$  as  
a contributor to the carcinogenic process.

#### Materials and methods

15 Cell lines. The LNCaP cell line was derived from  
metastatic deposits from a patient with advanced prostate  
cancer (11) and was provided by Dr. Steven Harris (W.  
Alton Jones Cell Science Center, NY). CREF-Trans 6 and  
LNCaP DNA-transfected nude mouse tumor-derived CREF-Trans  
20 6 cells, CREF-Trans 6:4 NMT, were isolated as described  
previously (12). The hormone independent prostatic  
carcinoma cell line DU-145, the endometrial carcinoma  
cell line HTB-113, the small cell lung carcinoma cell  
line NCI-H69 and the human neuroblastoma cell line IMR-32  
25 were obtained from the American Type Culture Collection.  
The nasopharyngeal carcinoma cell line KB 3-1 was  
provided by Dr. Michael M. Gottesman (NCI, MD). The human  
breast carcinoma cell line MCF 7 and the human colon  
carcinoma cell lines WiDr, HT 29, SW480 and LS174T were  
30 supplied by Dr. John W. Greiner (NCI, MD). The human  
breast carcinoma cell line T47D was provided by Dr.  
Ricardo Mesa-Tejada (MetPath Inc., NJ). A normal human  
cerebellum cell line, a human glioblastoma multiforme  
cell line GBM-18 and a human neuroblastoma cell line NB-  
35 11 were established in the applicants' laboratory (17-



-67-

- 19). H0-1 human melanoma cells were obtained from Dr. Beppino Giovannella (Stehlin Foundation, TX). C8161 metastatic human melanoma cells were supplied by Dr. Danny R. Welch (Hershey Medical Center, PA). The human
- 5 osteosarcoma cell line Saos-2 was provided by Dr. C. S. Hamish Young (Columbia Univ., NY). Conditions for growing the various cell types were as described previously (11,12,17-19).
- 10 RNA preparation, differential RNA display (DD) and RT-PCR. Total cytoplasmic RNA was isolated from logarithmically growing cell cultures as previously described (14,15). Tissue samples from normal prostates
- 15 and patients with prostatic carcinomas or BPH were frozen in liquid nitrogen and RNA was isolated using the TRIzol reagent as described by GibcoBRL (MD). Tissue samples were supplied by the Cooperative Human Tumor Network (CHTN). Samples of normal prostate were obtained from
- 20 autopsies of males < 40 years of age. All tissues were histologically confirmed as normal, BPH or carcinoma of the prostate. The DD procedure was performed essentially as described by Liang and Pardee (13). Two  $\mu$ g of mRNAs from CREF-Trans 6 and CREF-Trans 6:4 NMT cells were reverse transcribed with 300 units of MMLV reverse
- 25 transcriptase (BRL) in the presence of 2.5  $\mu$ M of primer T12GC (5'-TTTTTTTTTTTGC-3') and 20  $\mu$ M dNTP mix (BRL) for 60 min at 35°C. Two  $\mu$ g of the cDNA was PCR-amplified in the presence of 2  $\mu$ M T12GC and 2  $\mu$ M of a 5'-primer JB-24 (5'-ACCGACGTCGACTATCCATGAACA-5'). Samples were resolved
- 30 in parallel lanes on a 5% denaturing sequencing gel and differentially expressed bands were removed from the gel and electroeluted in 0.2 X TBE solution. The same pair of primers were used for PCR amplification of the differentially expressed sequences followed by TA cloning
- 35 kit (Invitrogen). Plasmids containing inserts of the

-68-

predicted size were sequenced by the Sanger method (Sequenase kit, version 2.0, USB) or the inserts were isolated and used to probe Northern blots (14-16). RT-PCR using appropriate primers was performed as described previously (16).

**cDNA library construction, screening and sequencing.** A cDNA library of LNCaP mRNA was constructed in the Uni-ZAP XR vector (Stratagene) and screened as previously described (14). A 1.8 kb PTI-1 DNA fragment was obtained by RT/PCR amplification of LNCaP cDNA with a 20 mer (5'-AACTAAGTGGAGGACCGAAC-3') within the 214 bp DNA obtained by DD. Inserts from the plasmids containing the largest PTI-1 inserts were excised by digestion with the restriction enzymes XhoI and EcoRI and tested by Northern blotting with appropriate RNA samples and sequenced using the Sanger method with an Applied Biosystems (Model 373A, Version 1.2.1) sequencer and oligonucleotides synthesized from both ends of the gene inserts.

**RACE procedure.** To identify the 5'-extended region of PTI-1, a 22 base oligomer (I) (5'-CCTTG CATATTAACATAACTCG-3') and a 19 base oligomer (II) (5'-AAGTCGCCCTATT CAGACT-3'), antisense direction of the sequences 262-283 bp and 317-336 bp, respectively, were synthesized. The RACE protocol was performed using the 5' RACE system (GibcoBRL, MD) as previously described (14).

**In vitro translation of PTI-1 encoded proteins.** PTI-1 was linearized by digestion with XhoI and used as a template to synthesize mRNA using the mCAP mRNA capping kit (Stratagene). In vitro translation of PTI-1 was performed using a rabbit reticulocyte lysate translation kit with conditions as described by GibcoBRL (MD).

35

-69-

### Experimental Results

Identification and properties of PTI-1. The rapid expression cloning system identified a potential oncogenic element in LNCaP cells (12). To identify genes displaying differential expression in CREF-Trans 6 cells and nude mouse tumor-derived LNCaP-transfected CREF-Trans 6 cells, CREF-Trans 6:4 NMT, the DD approach developed by Liang and Pardee (13) was used. This protocol permits identification of differentially expressed cDNAs based on size as opposed to nucleotide composition or function. A problem often encountered using DD is the identification of amplified sequences not displaying differential expression when tested using appropriate RNA samples and Northern blotting (12). An example of this type of artifact is seen in Fig. 6, i.e., the band present in CREF-Trans 6:4 NMT directly below the arrow. The frequency of false signals can be significantly reduced by using subtraction hybridization prior to PCR amplification and DD (data not shown). Using DD, a 214 bp DNA fragment (PTI-1) was identified in the LNCaP-transfected nude mouse tumor-derived CREF-Trans 6 cell line, CREF-Trans 6:4 NMT, that was not present in parental CREF-Trans 6 cells (Fig. 6, arrow). The PTI-1 fragment was isolated, cloned, sequenced and used to probe Northern blots containing RNAs from CREF-Trans 6, CREF-Trans 6:4 NMT and LNCaP cells (Fig. 7). The 214 bp PTI-1 DNA fragment is a novel sequence and hybridizes to several RNAs present in CREF-Trans 6:4 NMT, LNCaP and the hormone independent prostate carcinoma cell line DU-145 (Fig. 7).

The complete sequence of PTI-1 is presented in Fig. 8. PTI-1 consists of 2,123 bp, the 5'-flanking region (1 to 215 bp) was obtained by RACE 5'-extension and the

-70-

remainder of the gene (216 to 2,123 bp) was determined by direct sequencing. Primer extension analysis and RT-PCR of LNCaP mRNA confirm that PTI-1 is a full-length cDNA (data not shown). The 3' region of PTI-1 extending from  
5 630 to 2,123 bp displays 97% homology to a truncated human EF-1 $\alpha$  gene. The 5' region of PTI-1 displays no homology to eucaryotic genes, but instead is ~85% homologous to procaryotic 23S ribosomal RNA gene from *Mycoplasma hyopneumoniae*. This region of PTI-1 contains  
10 the 214 bp DNA marker (core) sequence obtained using DD (Fig. 6). The unique 5' region also contains a large number of stop codons (TAA, TGA and TAG sequences) (Fig. 8). These observations suggest that PTI-1 is a fusion gene consisting of two regions: a 5' unique 630 bp region  
15 and a 3' truncated and mutated EF-1 $\alpha$  gene.

PTI-1 contains an open-reading frame from bp 621 to 1,814 with a stop codon after the last amino acid K and encodes a protein of 398 aa (Fig. 8). A comparison of the amino  
20 acid sequence of PTI-1 (1 to 398 aa) and a partial human EF-1 $\alpha$  (aa 1 to 462) is presented in Fig. 8. PTI-1 and the truncated human EF-1 $\alpha$  share 98.4 % similarity and 97.7% identity. PTI-1 contains the same carboxyl terminus as human EF-1 $\alpha$ . The N-terminus of PTI-1 is different from  
25 human EF-1 $\alpha$  and consists of a deletion of 67 aa normally found in human EF-1 $\alpha$  and an insertion of 3 unique amino acids (MQS) in PTI-1 that differs from the original N-terminus (MGK) of human EF-1 $\alpha$ . In addition, 6 in frame amino acid changes are present in PTI-1 (Fig. 8). The  
30 loss of 67 amino acids in the N-terminus plus changes in specific amino acids, from positive charged to non-positive charged amino acids and from hydroxyl group-containing to non-hydroxyl group-containing amino acids, can be anticipated to impact on the three dimensional  
35 structure and functionality of this mutant EF-1 $\alpha$  protein.

-71-

On the basis of sequence analysis, PTI-1 should encode a protein of 43.8 kDa. To confirm this prediction, in vitro translation analyses of proteins encoded by the PTI-1 cDNA were determined (Fig. 9). A predominant protein present after in vitro translation of PTI-1 has an  $M_r$  of ~46 kDa. This value is larger than predicted and may result because of protein modification, i.e., phosphorylation, in the rabbit reticulocyte lysate system. Four additional minor proteins ( $M_r$  41 to 30.5 kDa) are also present after in vitro translation. These proteins probably result from initiation of protein synthesis at start codons (ATG) downstream of the first start codon in PTI-1 (Fig. 8).

**Expression of PTI-1 in RNA samples from patient-derived tissues and cell lines.** An important question is whether PTI-1 expression occurs in prostatic carcinomas in vivo. For this analysis, RNA was isolated from quick-frozen prostate samples from patients obtained during operations and confirmed as prostatic carcinomas or BPH histologically. RNAs were also extracted from normal prostates obtained at autopsy from men less than 40 years of age and histologically normal. Using RT-PCR with primers (A and L) (Fig. 8) synthesized from the unique 630 bp 5' PTI-1 sequence, expression is apparent in seven of eight human prostatic carcinomas (Fig. 10 and data not shown). In contrast, PTI-1 is not expressed in four normal prostates or three BPH patient samples (Fig. 10). In contrast, LNCaP and all prostate samples, including normal, BPH and carcinoma, are positive for PSA expression, whereas CREF-Trans 6, CREF-Trans 6:4 NMT and DU-145 do not express PSA (Fig. 10). All samples were positive for GAPDH expression (Fig. 10). These results indicate that PTI-1 is expressed in human prostate carcinomas, but not in normal prostates or BPH.

-72-

To determine the pattern of PTI-1 expression in additional cell types, RNAs from various cell lines were analyzed by Northern blotting using PTI-1 and GAPDH as probes (Fig. 7). In addition to being expressed in CREF-Trans 6:4 NMT, LNCaP and DU-145, PTI-1 expression is evident in other human carcinomas, including NCI-H69 (small cell lung), T47D (breast), and SW480 and LS174T (colon). In contrast, PTI-1 expression is not detected in HTB-113 (endometrial adenocarcinoma), KB 3-1 (nasopharyngeal carcinoma), MCF 7 (breast carcinoma), WiDr and HT 29 (colon carcinoma), normal cerebellum, GBM-18 (glioblastoma multiforme), H0-1 and C8161 (melanoma), NB-11 and IMR-32 (neuroblastoma) or Saos-2 (osteosarcoma) cells. These observations indicate that PTI-1 expression is not restricted to human prostate carcinoma, but also occurs in ~50% of the human carcinomas analyzed.

#### Experimental Discussion

Cancer is a progressive disease in which tumor cells manifest continuous genetic changes that correlate with increasing frequencies of chromosomal abnormalities and mutations (rev. 20-22). Recent studies suggest that mutations in genes involved in maintaining genomic stability, including DNA repair, mismatch repair, DNA replication and chromosomal segregation, may result in acquisition of a mutator phenotype by cancer cells predisposing them to further mutations resulting in tumor progression (rev. 21). In leukemias as well as specific solid tumors, improved cytogenetic techniques and molecular approaches indicate that specific translocations result in the activation of proto-oncogene products and the creation of tumor-specific fusion proteins (22). A common observation is that both types of novel oncogenic elements are often transcription

-73-

factors suggesting that alterations in transcriptional control may directly contribute to cancer development and evolution (22,23). Modifications in the translational machinery of cells, including changes in both eucaryotic

5 initiation factors and elongation factors, can also result in susceptibility to transformation and the acquisition of transformed and oncogenic properties in specific target cells (rev. 24,25). For example, overexpression of a normally rate-limiting protein

10 initiation factor, eIF-4E, can cooperate with both the v-myc and adenovirus E1A gene in inducing transformation of primary rodent fibroblasts (26), induce tumorigenic transformation in both NIH 3T3 and Rat 2 cells (27) and induce in combination with Max both a tumorigenic and

15 metastatic phenotype in Chinese hamster ovary (CHO) cells (28). Enhanced expression of elongation factor-1 $\alpha$  (EF-1 $\alpha$ ), a nucleotide exchange protein that binds GTP and aminoacyl-tRNA and results in codon-dependent placement of this aminoacyl-tRNA at the A site of the ribosome

20 (24,25), confers susceptibility to carcinogen- and ultraviolet light-induced transformation to mouse and Syrian hamster cell lines (29). Elevated levels of wild-type EF-1 $\alpha$  also occur in tumors of the pancreas, colon, breast, lung and stomach relative to normal tissue (30).

25 Moreover, enhanced expression of EF-1 $\gamma$ , a nucleotide exchange protein that mediates transport of aminoacyl tRNAs to 80S ribosomes during RNA translation, is found in a high proportion of pancreatic tumors (78%), colorectal tumors (86%) and colorectal adenomas (56%)

30 relative to normal-appearing adjacent tissue (31-33). These findings indicate that alterations in both gene transcription and protein synthetic processes contribute to oncogenesis.

35 The present study implicates a novel gene, PTI-1, that

-74-

contains a unique sequence linked to a truncated and mutated EF-1 $\alpha$  gene, in oncogenic transformation and prostate carcinoma development. PTI-1 is expressed in LNCaP-transfected tumor-derived CREF-Trans 6 cells, human  
5 prostatic carcinoma cell lines and patient-derived carcinomas, whereas expression is not detected in normal prostate or BPH tissues. PTI-1 RNA is also found in additional human carcinomas of the breast, lung and colon. These results indicate that PTI-1 expression may  
10 be a common alteration in human carcinomas. The direct cloning of PTI-1 from an LNCaP cDNA library indicates that this novel gene is originally present in this prostatic carcinoma cell line and does not develop as a consequence of mutation resulting during transfection  
15 into CREF-Trans 6 cells or selection for tumor-formation in nude mice.

EF-1 $\alpha$  is analogous to bacterial elongation factor-Tu (EF-Tu), both members of the GTPase superfamily of proteins  
20 (rev. 34-36). A primary function of EF-Tu/EF-1 $\alpha$  is the process of kinetic proofreading that results in appropriate codon-anticodon binding interactions (36). Mutations in specific regions of EF-Tu result in altered biological function, including a dominant negative  
25 inhibition of protein synthesis by mutational replacement of Lys 136 by glutamate or glutamine in the G-4 GTPase region that interacts with guanine nucleotide release proteins (GNRPs) (37). EF-Tu mutants in *Escherichia coli* and *Salmonella* exhibit increases in missense error rates  
30 (38,39). Mutations in EF-1 $\alpha$  can directly affect the frequency of frameshifting and amino acid misincorporations in *Saccharomyces cerevisiae* (40). Single amino acid substitutions in EF-1 $\alpha$  alter the selection and/or proofreading of the codon-anticodon  
35 match (40). Moreover, altering the level of EF-1 $\alpha$  in



-75-

*Saccharomyces cerevisiae* directly affects suppression of nonsense mutations further indicating a critical involvement in translational fidelity (41). In this context, the mutated EF-1 $\alpha$  protein encoded by PTI-1 could  
5 modify normal EF-1 $\alpha$  function resulting in decreased protein translational fidelity and an inability to suppress specific mutations in carcinomas. If this "translational infidelity" hypothesis is correct, PTI-1 may represent a mutated "genomic stability" gene (21)  
10 and an important contributor to the mutator phenotype of cancer cells and tumor progression.

An important early event in carcinogenesis may involve mutations that confer immortality or an enhanced cellular  
15 life span (20,21). During cellular senescence the levels and catalytic activity of EF-1 $\alpha$  decrease (42). Forced expression of EF-1 $\alpha$  in *Drosophila melanogaster* extends life-span in comparison with control flies (43). The reduction in proliferative capacity associated with  
20 senescence correlates with a reduced capacity for mitosis. In this respect, the recent demonstration that EF-1 $\alpha$  may be an important element in mitotic spindle formation (44) may be relevant. As demonstrated in this report, the EF-1 $\alpha$  sequence in PTI-1 contains a deletion  
25 of 67 amino acids and six point mutations in comparison with wild-type human EF-1 $\alpha$  (Fig. 8). Although the relevance of these alterations to EF-1 $\alpha$  activity are unknown, it is possible that this gene undergoes a series of step-wise mutations during prostate cancer  
30 development. If this hypothesis is correct, changes in the structure of the PTI-1 gene could represent a genetic marker for prostatic carcinoma development and progression. Studies are currently in progress to test these hypotheses and to determine if expression of PTI-1  
35 and/or genetically modified EF-1 $\alpha$  genes in CREF-Trans 6

-76-

cells results in acquisition of oncogenic potential.

5 A previous limitation preventing the identification and  
cloning of novel oncogenes was the absence of a sensitive  
transfectable indicator cell line. This problem has been  
ameliorated with the identification of the CREF-Trans 6  
clone (12). Using rapid expression cloning with the CREF-  
Trans 6 acceptor cell line and the DD technology, the  
10 novel putative oncogene PTI-1 displaying expression in  
human prostate, breast, lung and colon carcinomas has  
been identified and cloned. In comparative studies using  
NIH-3T3 cells, cotransfection of high molecular weight  
DNA from LNCaP cells and antibiotic resistance plasmid  
15 (pSV2neo) DNA did not result in tumors following  
injection of G418-resistant cells into nude mice (12).  
Rapid expression cloning with CREF-Trans 6 also results  
in the transfer of tumor-inducing oncogenes from a human  
breast carcinoma, a glioblastoma multiforme and a small  
20 cell lung carcinoma cell line and from a patient-derived  
metastatic colon carcinoma lesion (data not shown).  
Although the identifications of the dominant-acting  
genetic elements present in these human tumor DNA-  
transfected CREF-Trans 6 clones are not known, these  
25 exciting preliminary results suggest that this new  
acceptor cell line could prove useful for identifying and  
cloning potentially novel human oncogenes involved in the  
development of diverse human cancers.

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References of the Second Series of Experiments

1. Garnick, M.B. (1994) *Scient. Amer.* 270, 72-81.
- 5 2. Epstein, J.I., Pizov, G. & Walsh, P.C. (1993) *Cancer* 71, 3582-3593.
3. Jewett, H.J., Bridge, R.W., Gray, J.F., Jr. & Shelly, W.M. (1968) *JAMA* 203, 403406.
4. Mukamel, E., Hanna, J. & deKernion, J.B. (1987)  
10 *Urol.* 30, 318-323.
5. Salo, J.O., Kivisaari, L., Rannikko, S. & Lehtonen, T.J. (1987) *Urol.* 137, 435-438..
6. Hricak, H., Doms, G.C., Jeffrey, R.B., Arallone, A., Jacobs, D., Benton, W.K., Narayan, P. & Tanagho, E.A. (1987) *Radiology* 162, 331-336.  
15
7. Anscher, M.S. & Prosnitz, R. (1987) *J. Urol.* 138, 1407-1412.
8. Lu-Yao, G.L., McLerran, D., Wasson, J. & Wennberg, J.E. (1993) *JAMA* 269, 2633-2655.
- 20 9. Barbacid, M. (1987) *Ann. Rev. Biochem.* 56, 779-827.
10. Fasano, O., Birnbaum, D., Edlund, L., Fogh, J. & Wigler, M. (1984) *Mol. Cell. Biol.* 4, 1695-1705.
11. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Ming Chu, T., Mirand, E.A. & Murphy, G.G. (1983) *Cancer Res.* 43, 1809-1818.  
25
12. Su, Z.-z., Olsson, C.A., Zimmer, S.G. & Fisher, P.B. (1992) *Anticancer Res.* 12, 297-304.
13. Liang, P. & Pardee, A.B. (1992) *Science* 257, 967-971.
- 30 14. Jiang, H., Lin, J. & Fisher, P.B. (1994) *Mol. Cell. Different.* 2 (3), 221-239.
15. Jiang, H., Lin, J., Su, Z.-z., Herlyn, M., Kerbel, R.S., Weissman, B.E., Welch, D.R. & Fisher, P.B. (1995) *Oncogene*, in press.
- 35 16. Lin, J., Su, Z.-z., Grunberger, D., Zimmer, S.G. &

-78-

- Fisher, P.B. (1994) *Intl. J. Oncol.*, 5, 5-15.
17. Vita, J.R., Edwalds, G.M., Gorey, T., Housepian, E., Fetell, M.R., Guarini, L., Langer, J.A. & Fisher, P.B. (1988) *Anticancer Res.* 8, 297-302.
- 5 18. Guarini, L., Temponi, M., Bruce, J.N., Bollon, A.P., Duigou, G.J., Moulton, T.A., Ferrone, S. & Fisher, P.B. (1990) *Int. J. Cancer* 46, 1041-1047.
19. Reddy, P.G., Graham, G.M., Datta, S., Guarini, L., Moulton, T.A., Jiang, H., Gottesman, M.M. & Fisher, P.B. (1991) *J. Natl. Cancer Inst.* 83, 1307-1315.
- 10 20. Fisher, P.B. (1984) in *Tumor Promotion and Cocarcinogenesis In Vitro, Mechanisms of Tumor Promotion*, ed. Slaga, T.J. (CRC Press, Florida), pp. 57- 123.
- 15 21. Loeb, L.A. (1994) *Cancer Res.* 54, 5059-5063.
22. Rabbitts, T.H. (1994) *Nature* 372, 143-149.
23. Su, Z.-z., Austin, V.A., Zimmer, S.G. & Fisher, P.B. (1993) *Oncogene* 8, 1211-1219.
24. Riis, B., Rattan, S.I.S., Clark, B.F.C. & Merrick, W.C. (1990) *TIBS* 15, 420-424.
- 20 25. Sonenberg, N. (1993) *Current Biol.* 5, 955-960.
26. Lazaris-Karatzas, A. & Sonenberg, N. (1992) *Mol. Cell. Biol.* 12, 1234-1238.
27. Lazaris-Karatzas, A., Montine, K.S. & Sonenberg, N. (1990) *Nature* 345, 544-547.
- 25 28. De Benedetti, A., Joshi, B., Graff, J.R. & Zimmer, S.G. (1994) *Mol. Cell. Different.* 2 (4), 309-334.
29. Tatsuka, M., Mitsui, H., Wada, M., Nagata, A., Nojima, H. & Okayama, H. (1992) *Nature* 359, 333-336.
- 30 30. Grant, A.G., Flomen, R.M., Tizard, M.L.V. & Grant, D.A.W. (1992) *Int. J. Cancer* 51, 740-745.
31. Chi, K., Jones, D.V. & Frazier, M.L. (1992) *Gastroenterology* 103, 98-102.
32. Lew, Y., Jones, D.V., Mars, W.M., Evans, D., Byrd, D. & Frazier, M.L. (1992) *Pancreas* 7, 144-152.
- 35

-79-

33. Ender, B., Lynch, P., Kim, Y.H., Inamdar, N.V.,  
Cleary, K.R. & Frazier, M.L. (1993) *Mol. Carcinog.*  
7, 18-20.
- 5 34. Bourne, H.R., Sanders, D.A. & McCormick, F. (1990)  
*Nature* 348, 125-132.
35. Bourne, H.R., Sanders, D.A. & McCormick, F. (1991)  
*Nature* 349, 117-127.
36. Merrick W.C. (1992) *Microbiol. Rev.* 60, 291-315.
- 10 37. Hwang, Y.W., Sanchez, A. & Miller, D.L. (1989) *J.*  
*Biol. Chem.* 264, 8304-8309.
38. Tapio, S. & Kurland, C.G. (1986) *Mol. Gen. Genet.*  
205, 186-188.
39. Hughes, D., Atkins, J.F. & Thompson, S. (1987) *EMBO*  
*J.* 6, 4235-4239.
- 15 40. Sandbaken, M.G. & Culbertson, M.R. (1988) *Genetics*  
120, 923-934.
41. Song, J.M., Picologlou, S., Grant, C.M., Firoozan,  
M., Tuite, M.F. & Liebman, S. (1989) *Mol. Cell.*  
*Biol.* 9, 4571-4575.
- 20 42. Cavallius, J., Rattan, S.I.S. & Clark, B.F.C. (1986)  
*Exper. Gerontol.* 21, 149-157.
43. Shepherd, J.C.W., Walldorf, U., Hug, P. & Gehring,  
W.J. (1989) *Proc. Natl. Acad. Sci. USA* 86, 7520-  
7521.
- 25 44. Marchesi, V.T. & Ngo, N. (1993) *Proc. Natl. Acad.*  
*Sci. USA* 90, 3028-3032.

-80-

### Third Series of Experiments

5 The American Cancer Society estimates that 200,000 American men will have been diagnosed with prostate cancer in 1994 and 38,000 afflicted men will have died of this disease. The current methods for detecting early prostate cancer are limited in both their sensitivity and specificity. These include physical examination that might easily miss small or centrally located tumors, 10 serum prostate-specific antigen (PSA) determination that is not specific to malignant prostate disease, and tissue biopsy in which sampling error may lead to erroneous benign diagnosis. Predictors and early detection of therapeutic relapse such as monitoring of PSA levels, 15 ultrasound and bone scans are also unsatisfactory, as these require fairly bulky tumor regrowth before discovery.

20 Despite intensive scientific effort, the relevant genomic changes that mediate the development and evolution of prostate cancer remain to be defined. In addition, biochemical and molecular markers correlating with potential aggressiveness of a specific prostate carcinoma and the appropriate therapy that will effectively prevent 25 disease progression are not currently available. It is now well established that many forms of cancer are the result of complex multifactor interactions and carcinogenesis is a multistep process. Genetic factors contributing to carcinogenesis include dominant acting 30 oncogenes that promote the cancer phenotype and tumor suppressor genes that function as negative inhibitors of the cancer process. Our broad goals are to define prostate cancer in molecular terms and use this information to design better diagnostic tools and 35 therapies for this malignancy. To achieve these

-81-

objectives it will be necessary to identify and characterize genes that can both induce and inhibit this disease process. Once appropriate genetic mediators of human prostate cancer are identified this information will prove valuable for developing more effective diagnostic tools and ultimately for generating improved gene-based and immunologically-based therapies for this pervasive cancer.

#### 10 The Rapid Expression Cloning (RExCS) System

A primary objective of investigators interested in the etiology of human cancer is the identification of gene(s) within tumor cells with oncogenic potential. One procedure used to achieve this goal involves the transfer of high molecular weight (HMW) DNA isolated from established tumor cell lines or primary tumors into appropriate cell lines by calcium-mediated DNA-transfection, lipofection, electroporation or other approaches. Target cells are then examined for signs of morphological transformation, i.e., focus formation. A modification of this approach involves cotransfection of target cells with HMW DNA plus a selectable antibiotic resistance gene, such as pSV2neo, selection for antibiotic resistance and then injection of pooled antibiotic resistant cells into nude mice to identify clones of cells with tumorigenic potential. The majority of studies using these approaches have relied on the immortal murine cell line NIH-3T3. Unfortunately, NIH-3T3 cells have generally not proven successful in identifying novel dominant-acting oncogenes from human tumor lines or clinical samples and even when successful, subsequent cloning of the transforming gene has revealed genetic elements not relevant to most human cancers. These findings emphasize the need for improved techniques to

-82-

identify dominant-acting human cancer genes and the identification of more suitable target cell lines that can express novel tumor-inducing human oncogenes.

- 5 To identify dominant acting oncogenes in human prostate carcinoma cells applicants have used 2 approaches, both utilizing DNA cotransfection techniques with a new DNA-acceptor cell line, CREF-Trans 6, and tumor formation in nude mice as an endpoint. Cotransfection of CREF-Trans 6 with HMW DNA from the human prostate carcinoma cell line LNCaP and pSV2neo DNA, selection for G418 resistance and injection into nude mice resulted in tumor formation. In contrast, no transformed foci were apparent in similarly transfected CREF-Trans 6 cells maintained only in monolayer culture. No dominant acting focus forming or tumor inducing oncogene was detected in NIH-3T3 cells cotransfected with LNCaP and pSV2neo DNA. Both primary and secondary nude mouse tumor-derived CREF-Trans 6 cells contain human repetitive (Alu) sequences that are not present in untransfected CREF-Trans 6 cells. A common Alu fragment is present in Southern blots in both primary and secondary tumor derived CREF-Trans 6 cells. Tumor-derived CREF-Trans 6 cells also contain additional Alu sequences of different apparent molecular sizes. This data provided initial supportive evidence that a human gene(s) potentially capable of inducing a tumorigenic phenotype in nontumorigenic CREF-Trans 6 cells had been transferred from the human prostate carcinoma cell line LNCaP. Using both molecular and immunological approaches tumor-derived CREF-Trans 6 cells have been used to: (a) identify and clone novel genes, termed prostate tumor inducing genes (PTI-1, PTI-2, figure 13 and PTI-3, figure 14), potentially involved in the etiology of human prostate carcinoma; and (b) produce monoclonal antibodies reacting with the surface of human prostate carcinoma



-83-

cells and the cloning of a cDNA encoding a novel tumor associated antigen, termed prostate carcinoma tumor antigen gene (PCTA-1, figure 15).

5     Other Applications of RExCS: Cotransfection of CREF-Trans  
6 with pSV2neo DNA and high molecular weight DNA from a  
human glioblastoma multiforme cell line (GBM-18), a human  
breast carcinoma cell line (T47D) and a human small cell  
lung carcinoma cell line (NCI-H69) results in tumor  
10    formation in nude mice. Similarly, cotransfection of  
CREF-Trans 6 with pSV2neo DNA and high molecular weight  
DNA from a patient-derived metastatic colorectal  
carcinoma results in tumor formation in nude mice. Tumor-  
derived cell lines have been isolated and can now be  
15    used: to clone the transforming genetic elements  
mediating the tumorigenic phenotype; and with the SEM  
procedure to develop potentially novel MAbs reacting with  
TAAs expressed by specific human cancers.

20    Prostate Tumor Inducing Gene-1 (PTI-1): PTI-1 was  
initially identified in LNCaP DNA transfected tumor-  
derived CREF-Trans 6 cells using an approach termed RNA  
differential display (DD). DD permits the identification  
and cloning of differentially expressed mRNAs encoded by  
25    closely related cell types. The basic DD approach  
involves a series of interrelated steps, including: (a)  
isolating mRNA from two closely related cell types; (b)  
producing reverse transcribed-PCR (RT-PCR) products using  
a primer that anchors the PCR products to the 3' end of  
30    the mRNA and 5' primers containing arbitrary  
oligonucleotides of various sizes; (c) running RT-PCR  
products from both cell types in adjacent lanes of a  
sequencing gel; (d) cutting differentially expressed  
bands out of the sequencing gel, eluting the PCR product  
35    and PCR amplification; (e) testing for expression of the

-84-

PCR product using Northern blots containing relevant RNA samples; and (f) sequencing appropriately expressed sequences to determine identity with previously reported genes. Improvements in DD include the use of a subtraction hybridization step prior to performing RT-PCR. This technical improvement to DD results in a dramatic reduction in the number of false positives, that can exceed 40% using standard DD.

10 The DD cloning strategy has now been successfully used with untransfected CREF-Trans 6 cells and an LNCaP DNA transfected nude mouse tumor derived CREF-Trans 6 clone, CREF-Trans 6: 4 NMT to identify PTI-1 (PTI-2 and PTI-3- to be described below). An anchored oligo-dT primer  
15 consisting of 12 Ts plus two additional 3' bases, that provides specificity, was used to anneal the beginning of a subpopulation of the poly(A) tails of the mRNAs for reverse transcription. A set of arbitrary primers was used as a 5'-primer for PCR amplification of the cDNAs  
20 generated by reverse transcription from the mRNAs. These amplified cDNA fragments were then separated by size to a maximum of 500 bp on a denaturing polyacrylamide gel. A differentially expressed band of 214 bp that was present in CREF-Trans 6: 4 NMT but not in CREF-Trans 6  
25 was cut out of the gel, electroeluted in TBE and reamplified by PCR using the same primers as used for DD. The purified 214 bp DNA fragment from the 1% agarose gel was then cloned into the PCR<sup>TM</sup>II vector and transformed in OneShot<sup>TM</sup> competent cells. This sequence referred to as  
30 214 bp PTI-1, was tested for expression using Northern blotting analysis. PTI-1 was expressed in LNCaP and CREF-Trans 6: 4 NMT, but not in CREF-Trans 6, human breast carcinoma cells (MCF7), human glioblastoma multiforme (GBM-18) or human melanoma cells (H0-1 and C8161).  
35 Sequence analysis of the 214 bp PTI-1 DNA fragment using

-85-

the Sanger sequencing procedure indicates no homology to previously reported genes deposited in various gene banks (GenBank (R), Brookhaven Protein Data Bank, EMBL Data Library). In order to identify the 5'- and 3'-flanking regions of the 214 bp DNA, rapid amplification of cDNA ends (RACE) was performed. The RACE approach is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and an unknown sequence representing either the 3' or 5' end of the mRNA. Using RACE and primers designed from the 214 bp PTI-1 sequence, a 1.8 Kb PTI-1 DNA fragment was generated by PCR. Northern analysis using the 1.8 Kb PTI-1 DNA fragment produced the same reactivity pattern as observed with the 214 bp PTI-1 DNA fragment. Approximately 600 bp of the 5' region of the PTI-1 DNA fragment was sequenced and found to display no homology to reported eucaryotic gene sequences, but rather ~85% homology to 23S ribosomal RNA from Mycoplasma hyopneumoniae). This 1.8 Kb PTI DNA was called PTI-3 and will be described later. This 1.8 Kb PTI DNA was later used to screen an LNCaP cDNA library constructed in the Uni-ZAP XR vector. Two clones were identified. One is clone 18, which is called PTI-1; another, clone 8, is called PTI-2 and will be described later.

The PTI-1 gene is composed of two parts; one is 5'-RACE extended region (1-215 bp) and another clone 18 part. Clone 18 (PTI-1) contains 1937 bp (29 bp + 1908 bp) insert in pBluescript vector. The experiment demonstrates that the 29 bp at the 5'-end comes from wrong reverse transcription because of lower temperature and secondary structure of RNA, so that this 29 bp sequence was replaced by the right sequence obtained by RACE method and did not show in the complete sequence of PTI-1. The sequence from 30-1937 bp (1907 bp) of clone

-86-

18 was shown as the sequence 216 bp-2123 bp (1907 bp) in Figure 3. The plasmid deposit is clone 18 (PTI-1). A pair of oligonucleotides with sequences in the 5' region of the 1.8 Kb PTI-1 DNA was synthesized and RT-PCR was performed using mRNA isolated from both cell lines and human tissue samples. This analysis indicates that PTI-1 is expressed in CREF-Trans 6: 4 NMT, LNCaP, DU-145 (a hormone independent human prostate carcinoma cell line), 7 of 8 patient-derived prostate carcinomas. In contrast, PTI-1 is not expressed in CREF-Trans 6, H0-1, MCF-7 or tissue from normal human prostates or benign prostatic hypertrophy (BPH). These studies indicate that PTI-1 is a novel human oncogene that may be a mediator of or that is associated with transformation and tumorigenesis in human prostate carcinoma cells.

To identify a full-length PTI-1 cDNA, an LNCaP cDNA library was constructed in the Uni-ZAP XR vector. Screening the LNCaP library using the 1.8 Kb PTI-1 DNA probe resulted in the identification of an ~2.0 Kb PTI-1 cDNA from this library. Two approaches indicate that this PTI-1 cDNA is a full-length cDNA. One approach uses primer extension analysis of the ~2.0 Kb cDNA and the second approach involves in vitro translation of in vitro RNA transcribed from the ~2.0 Kb cDNA. Using a reticulocyte translation system, the transcribed RNA from the ~2.0 Kb PTI-1 cDNA generates several protein products with a predominant protein of approximately 46 kDa. Complete sequence analysis and comparison with existing DNA data bases of the ~2.0 Kb PTI-1 cDNA isolated from the LNCaP cDNA library indicates that PTI-1 is a novel fusion gene. PTI-1 consists of a unique 630 bp 5' sequence and a 3' sequence homologous to a truncated and mutated form of human elongation factor-1 alpha. A full-description of PTI-1 with sequence and specific

- 87 -

properties can be found in our PNAS paper.

PTI-1: Using primer sequences for bases present in the unique 630 bp 5' region of PTI-1 (A and L) and primer sequences corresponding to the elongation factor-1 alpha region of PTI-1 and RT-PCR approaches, the following additional information is currently available relative to PTI-1: (A) Tissue distribution studies (using tissue poly A<sup>+</sup> mRNA blots from Clontech) have been performed using the A and L primers and a region corresponding to the elongation factor-1 alpha homologous region of PTI-1 as probes. The unique region of PTI-1 is only expressed in skeletal muscle and colon tissue, whereas the elongation factor-1 alpha hybridizes with an mRNA present in all of the tissue samples. These include, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. These studies reinforce our previous observations that the unique region of PTI-1 is not expressed in normal human prostate. (B) Expression of PTI-1 (A and L primers) is reduced in LNCaP cells treated with: a phorbol ester tumor promoter (12-O-tetradecanoyl-phorbol -13-acetate (TPA), that induces apoptosis in LNCaP cells; suramin; epidermal growth factor; transforming growth factor-alpha; or the synthetic androgen R1881. Using primers for prostate specific antigen (PSA) reductions in PTI-1 mRNA levels using the same agents are also apparent in LNCaP cells. These results suggest that similar changes inducing downregulation of PSA expression can also decrease PTI-1 expression in human prostate carcinoma cells. (C) Expression is apparent in human promyelocytic leukemia (HL-60) and an additional leukemic cell line K562. When induced to differentiate by TPA, PTI-1 expression decreases and is no longer apparent by 3 hr

-88-

posttreatment in HL-60 cells. This change in mRNA levels after TPA treatment suggests that decreased expression of PTI-1 may be modulated as a function of growth arrest and terminal differentiation in HL-60 cells; (D) Expression is apparent in CREF cells transformed by diverse acting oncogenes, including wild-type 5 adenovirus (Ad5), mutant type 5 adenovirus (H5hr1), Ha-ras oncogene, v-src, human papilloma virus type 18 (HPV-18) and HPV-51. Using a dexamethasone (DEX) inducible Ad5 E1A transforming gene under the transcriptional control of a mouse mammary tumor virus promoter, expression of PTI-1 is only seen in the presence of DEX. Under these culturing conditions, DEX also results in E1A expression and transformation. These data indicate that induction of PTI-1 directly correlates with transformation induced by mechanistically different oncogenes. (Figure of Northern blot; Fig. 11).

Uses for PTI-1: The unique region of PTI-1 can be used to: (A) Produce primers for RT-PCR that will distinguish between prostate carcinoma and normal or BPH tissue samples (diagnostic applications); (B) Permit development of a blood test to identify prostate carcinoma cells that have metastatic potential and that have escaped from the prostate (diagnostic applications); (C) Permit identification of additional carcinomas, i.e., breast, colon and lung, in the blood stream that have resulted from metastatic carcinoma spread (diagnostic applications); and (D) Develop an antisense vector and/or ribozyme approach to inhibit expression and induce growth arrest and/or apoptosis in prostate carcinoma cells, and perhaps other carcinoma and leukemic cells (therapeutic applications).

The mutated elongation factor 1-alpha region of PTI-1 may prove useful to: (A) Identify genetic changes in cells

-89-

predisposing to carcinoma development and progression (diagnostic applications); (B) Identify point mutations and or deleted regions of this gene that could prove useful for predicting carcinoma development and progression (diagnostic applications); and (C) Develop antisense and ribozyme strategies to inhibit expression of the mutant form of elongation factor 1-alpha resulting in suppression of carcinoma growth (therapeutic applications).

10

PTI-1 and Prostate Tumor Inducing Gene-2 (PTI-2): A cDNA library was prepared from LNCaP cells in the Uni-ZAP XR vector (Stratagene). The LNCaP cDNA library was screened with a <sup>32</sup>P-labeled 1.8 Kb DNA (PTI-3) containing the 214 bp DNA obtained by the differential display procedure. The contents of ten plates of 150 mm X 15 mm (containing ~ 2 X 10<sup>4</sup> plaques/plate) were transferred in duplicate to nylon membranes. Hybridization was performed using the following conditions: 5% dextran sulfate, 45% deionized formamide, 4X SSC, 1 mM phosphate buffer (pH 7.5), 0.5% SDS, 5% Denhardt's reagent at 42°C in a Hybridization Incubator Model 400 (Robbin Scientific); washing at 55°C for 60 min in a solution of 0.25% SDS and 1X SSC. Positive plaques obtained in the first round were screened in duplicate for a second round and then with in vivo excision produced plasmids containing gene inserts in the pBluescript vector. Plasmids containing the longest inserts were identified by Southern blotting and probing with the 1.8 Kb (PTI-3) DNA probe. Two clones were identified using this approach: clone 8 (PTI-2) and clone 18 (partial sequence of PTI-1).

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The full sequence of PTI-1 contains 2,123 bp, the 5'-flanking (1-215 bp) was obtained by RACE 5'-extension (GIBCO-BRL), the remaining 216-2,123 bp was obtained by

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-90-

sequencing clone 18. The RACE 5' extension was performed with two oligonucleotides, both located within the 5'-end of clone 18. One oligonucleotide is a 23 mer (5'-CCTTGCATATTAACATAACTCGC-3') and the other oligonucleotide is a 20 mer (5'-AAGTCGCCCTATTTCAGACTC-3'). A comparison of PTI-1 with GenBank indicates that the 3'-part of this gene (630 to 2,123 bp) has 97% homology to human elongation factor 1-alpha. The 5'-part of this gene (1-629 bp) does not show any homology to known eucaryotic genes.

Comparison of PTI-2 with Genbank indicates that it has 86.9% identity in 1356 bp overlap with Mycoplasma floccular 16S ribosomal RNA and 23S ribosomal RNA genes, but no homology to any previously identified eucaryotic genes.

**Prostate Tumor Inducing Gene-3 (PTI-3)**: To identify genes specifically expressed in CREF-Trans 6:4 NMT (transformed by DNA from LNCaP cells), but not CREF-Trans 6 cells applicants have used the differential RNA display procedure. This approach resulted in the identification of a 214 bp DNA fragment in CREF-Trans 6:4 NMT that was not present in CREF-Trans 6 cells (PNAS paper). Northern blotting indicates that this 214 bp DNA is expressed in CREF-Trans 6:4 NMT and LNCaP cells, but not in CREF-Trans 6 cells. A 20 mer oligonucleotide with the sequence 5'-AACTAACTGGAGGACCGAAC-3' within this 214 bp DNA fragment was used to obtain extended sequences beyond the 214 bp DNA using the RACE method. A cDNA from LNCaP cells was synthesized with oligodT. To the 3'-end a polydC was added by terminal deoxynucleotide transferase. When the anchor primer (using the protocol of the GIBCO-BRL 5' RACE kit) and the above 20 mer were used to perform PCR amplification of cDNA from LNCaP cells, a 1.8 Kb DNA



-91-

fragment containing a partial sequence of the 214 bp DNA was obtained. This 1.8 Kb DNA fragment displays the same Northern blotting pattern as does the unique 214 bp sequence.

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The 1.8 Kb DNA fragment was cloned into PCR™ II vector by using the TA cloning kit (Invitrogen). The sequence of this 1.8 Kb DNA was determined by Sanger's method (Sequenase kit, version 2.0 USB). The 1.8 Kb DNA  
10 contains a partial sequence of PTI-1/3. The 5' and 3' end of PTI-3 gene remains to be confirmed. The insert of PTI-3 1.8 Kb insert can be recovered from the PCR™ II vector by digestion with EcoRI. A comparison of the sequence of PTI-3 with Genbank data base indicates that  
15 this gene has 87% identity in 1858 bp overlap with Mycoplasma floccular 16S ribosomal RNA and 23S ribosomal RNA genes, and it has 89.8% identity in 1858 bp overlap with Mycobacterium hyopneumoniae 23S ribosomal RNA gene.

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Prostate Carcinoma Tumor Antigen Gene-1 (PCTA-1):  
Evidence that tumor derived CREF-Trans 6 cells transfected with LNCaP DNA encode genetic information related to human prostate cancer has been obtained using  
25 an approach termed surface epitope masking (SEM). The SEM procedure involves the selective blocking of surface antigens present in a genetically engineered cell (referred to as a "tester") with high-titer polyclonal antibodies against the untransfected parental cell  
30 (referred to as a "driver"). Surface-epitope-masked tester cells are injected into BALB/c mice, immune spleen cells are then taken from these mice and they are fused with myeloma cells. This process results in the efficient generation of hybridomas that secrete  
35 monoclonal antibodies (MAbs) that react with cell-surface

-92-

antigens on transfected tester cells and with additional cell types that express the same surface molecules. LNCaP transfected tumor-derived CREF-Trans 6 cells, CREF-Trans 6: 4 NMT, have been used as a tester cell line. The SEM procedure was applied resulting in the development of hybridomas producing MAb's reacting with tumor associated antigens (TAAs) on the surface of the original LNCaP cell line used to obtain human prostatic carcinoma DNA, primary and secondary nude mouse transfectants derived from tumors and two additional human prostatic carcinoma cell lines (DU-145 and PC-3). These MAb's are designated Pro 1.1 to 1.5. Specific MAb's also display reactivity to two human breast carcinoma cell lines (MCF7 and T47D). However, they do not react with normal human skin fibroblasts (NH3F-1), two colon carcinoma cell lines (WiDr and LS174T), two human melanoma cell lines (H0-1 and MeWo) or a human glioblastoma multiforme cell line (GBM-18). Immunoprecipitation analyses of <sup>35</sup>S-methionine labeled cell extracts with PCTA-1 Pro 1.5 MAb's indicate that primary and secondary nude mouse tumor-derived LNCaP-transfected CREF-Trans 6 cells, LNCaP and DU-145 cells contain an approximately 42 kDa protein that is not present in untransfected CREF-Trans 6 or additional human tumors (including melanoma and glioblastoma multiforme). These results indicate that a gene encoding human prostatic carcinoma (and possibly breast carcinoma) TAAs has been transferred and is now expressed in CREF-Trans 6 cells.

To identify the gene encoding PCTA-1, a SEM-derived MAB (Pro 1.5) was used to screen an LNCaP cDNA expression library (PicoBlue Immunoscreening Kit, STRATAGENE). mRNA was isolated from LNCaP cells after passage of total RNA through an oligo-dT column (GIBCO). LNCaP cDNA libraries were constructed in the Uni-ZAP vector (Stratagene).

-93-

Screening of the cDNA library was performed as follows:

- (1) SURE host cells were plated on fifteen 150 mm X 15 mm NZY plates with 6.5 ml of top agar ( $\sim 2 \times 10^6$  plaques/plate);
- (2) After 3.5 hr incubation at 42°C,
- 5 nitrocellulose filters soaked with 10 mM IPTG solution were applied to the plates and plaques were lifted;
- (3) The filters containing the plaque lifts were washed 3 or 4 X with TBST (20 mM Tris-HCl pH7.5, 150 mM NaCl, 0.05 % Tween-20) and soaked in blocking solution (1% BSA in TBS
- 10 [20 mM Tris-HCl pH 7.5, 150 mM NaCl]) for 1 hr at room temperature;
- (4) The filters were then transferred to fresh blocking solution containing Pro 1.5 ascites (1:500 dilution) followed by incubation for 3 hr at room temperature with gentle rocking;
- (5) Filters were washed
- 15 4 X with TBST buffer;
- (6) The filters were transferred into fresh blocking solution containing Ab-AP conjugate (1:2000 dilution) and incubated for 1 hr at room temperature;
- (7) The filters were washed 4 X with TBST and placed in a developing solution containing 0.3 mg/ml
- 20 NBT (nitro blue tetrazolium, 0.15 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate), 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM  $MgCl_2$ ); and
- (8) The reaction was terminated with stop solution containing 20 mM Tris-HCl, pH 2.9 and 1 mM EDTA) and the filters were dried. In the
- 25 first round of screening with Pro 1.5, only 2 positive clones were obtained from over  $3 \times 10^6$  ( $15 \times 2 \times 10^4$ ) colonies. Screening was performed a second time and clones were isolated and characterized. Using this approach antibody-positive clones were identified that
- 30 contain a cDNA insert of  $\sim 3.8$  Kb. Sequence analysis of PCTA-1 indicates no homology to previously identified genes. The 5' region of PCTA-1 is homologous to several expressed sequence tags [including Homo sapiens partial cDNA sequence clone HEC077, clone c-zvh01, clone hbc1127
- 35 (3' end), clone hbc1208 (5' end) and clone hbc1074 (3'

-94-

end)] (see below). In vitro translation in a rabbit reticulocyte lysate system, with and without immunoprecipitation with Pro 1.5, indicate the presence of an approximately 36 kDa protein. These observations indicate that the PCTA-1 cDNA encodes a protein that is the putative tumor associated antigen present on prostate cancer cells identified using the SEM approach.

Comparison of PCTA-1 with Homo sapiens cDNA clones

10	Identity	PCTA-1	<u>Homo sapiens</u> partial cDNA sequence
		HEC077	c-zvh01 hbc 1127 hbc1208 hbc1074
			(3' end) (5' end) (3' end)

	94.9 %	2123/1732	1-395	
15	99 %	2118/1858		1-261
	88.8 %	3853/3562		1-290
	93.5 %	2630/2818		1-186
	84 %	2728/2825		1-98

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(1) SEM-Derived MAbS Specific for the Multidrug Resistance (MDR) P-Glycoprotein encoding an 170,000 Molecular Weight Cell Surface Transport Protein: To develop MAbS specific for the P-glycoprotein mediating MDR, CREF-Trans 6 cells were transfected with a human MDR-1 gene and cells resistant to colchicine were isolated. These MDR clones contain the MDR gene, express MDR mRNA and are cross-resistant to toxicity induced by several chemotherapeutic agents. MDR-CREF-Trans 6 cells were coated with CREF-Trans 6 polyclonal antibodies, injected into BALB/c mice, spleens were isolated and used to from hybridomas. Hybridomas secreting MAbS specific for MDR-CREF-Trans 6 cells were isolated. These SEM-derived MAbS react with MDR-CREF-Trans 6 cells as demonstrated by fluorescence activated cell sorter (FACS)

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-95-

analysis, confirming their interaction with epitopes of the P-glycoprotein expressed on the cell surface. In addition, human breast carcinoma (MCF-7) cells transfected with the same MDR-1 gene and displaying the MDR phenotype also react with the SEM-derived MABs. In contrast, non-MDR parental MCF-7 cells do not react with these MABs. These results indicate that the SEM approach can be used to develop MABs specific for defined cell surface-expressed molecules. (Full details in our JNCI manuscript- Shen, Su, Olsson, Goldstein & Fisher).

(2) SEM-Derived MABs Specific for the Human Leukocyte Interferon  $\alpha$  (IFN- $\alpha$ ) Receptor: To develop MABs specific for the Human IFN- $\alpha$  receptor, CREF-Trans 6 cells were transfected with a human IFN- $\alpha$  receptor expression vector and clones expressing the receptor were isolated. These clones interacted with labeled IFN- $\alpha$ , whereas non-transfected CREF-Trans 6 cells do not react with IFN- $\alpha$ . These results provide further documentation of the effectiveness of the SEM approach in producing MABs specific for defined cell surface-expressed molecules.

(3) SEM-Derived MABs Specific for the Human Immune Interferon (IFN- $\gamma$ ) Receptor: To develop MABs specific for the Human IFN- $\gamma$  receptor, CREF-Trans 6 cells were transfected with a human IFN- $\gamma$  receptor expression vector and clones expressing the receptor were isolated. These clones interacted with labeled IFN- $\gamma$ , whereas non-transfected CREF-Trans 6 cells do not react with IFN- $\gamma$ . These results provide further documentation of the effectiveness of the SEM approach in producing MABs specific for defined cell surface-expressed molecules.

(4) SEM-Derived MABs Reacting with Human Prostate Carcinomas: To determine if CREF-Trans 6 cells containing

-96-

a putative human prostate tumor inducing gene(s), CREF-Trans 6:4 NMT, display tumor associated antigens (TAAs) also expressed on human prostate carcinoma cells, the SEM approach has been used. This procedure and the experimental results using the CREF-Trans 6:4 NMT clone is described in our JNCI manuscript (Shen et al., JNCI 86: 91-98, 1994). The SEM-derived Pro MAb (Pro 1.1, Pro 1.2, Pro 1.3, Pro 1.4 and Pro 1.5) display reactivity with LNCaP cells as well as two additional human prostate carcinomas, DU-145 and PC-3. Specific Pro MAb also display surface reactivity with two human breast carcinoma cell lines, T47D and MCF-7. These MAb are now being tested for reactivity using in situ immunohistochemistry with sections obtained from patients with prostate cancer. The ability to generate these Pro MAb by SEM indicate that this approach can also be used to produce MAb specific for cell surface expressed molecules of unknown origin. The Pro 1.4 MAb have also been used in combination with expression cloning and human prostate carcinoma library screening to identify and clone the gene encoding the specific TAAs, PCTA-1.

(5) SEM-Derived MAb Reacting with Human Breast Carcinomas: To determine if CREF-Trans 6 cells containing a putative human breast carcinoma tumor inducing gene(s), CREF-Trans 6:T47D NMT, display TAAs also expressed in human breast carcinoma cells, the SEM approach has been used. This approach resulted in the development of SEM-derived Br-car (breast carcinoma) MAb (4.2.1 and 5.2.4) that react with T47D and MCF-7 human breast carcinoma cell lines. In situ immunohistochemistry (total of 10 samples) indicate that the SEM-derived Br-car MAb also react with carcinoma sections from patients with ductal and medullary breast carcinomas (Fig. 12). These MAb are negative in sections of human melanoma and a small cell

-97-

lung carcinoma (Fig. 12). The ability to generate these Br-car MAbs by SEM provide additional evidence that this approach can be used to produce MAbs specific for cell surface expressed molecules of unknown origin.

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**Potential Applications for SEM-Approach and SEM-Derived**

**MAbs**: (A) The SEM-approach represents a general strategy for producing MAbs specific for molecules expressed on the cell surface. These can include, but are not limited to, novel TAAs, growth factor receptors, T-cell reactive epitopes, cell surface antigens (representing different developmental stages), surface expressed oncogene products, viral encoded proteins found on the cell surface, surface antigens expressed as a function of tumor progression (e.g., antigens associated with benign disease and metastatic disease), surface antigens eliciting reactivity with non-specific immunoreactive cells (i.e., NK cells and macrophages) and surface antigens eliciting autoimmune diseases (diagnostic and

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therapeutic applications); (B) SEM-derived MAbs can be used for in situ immunohistochemistry to identify specific cell surface expressed molecules, including growth factor receptors, cell surface antigens, surface expressed oncogene products, TAAs and viral encoded proteins found on the cell surface (diagnostic applications); (C) SEM-derived MAbs can be used to target toxins and radionuclides to tumor cells (therapeutic applications); (D) SEM-derived MAbs with high reactivity toward specific clinically relevant target molecules can be used to develop chimerized (human-mouse) and humanized MAbs for both diagnostic applications and therapeutic applications in humans; (E) SEM-derived MAbs can be used to clone genes encoding TAAs and additional cell surface expressed molecules of unknown structure. These genes can then be used for diagnostic applications and ultimately

-98-

therapeutic applications; (E) SEM-derived MAbs can be used to identify potentially important TAAs. Once appropriate genes and antigens are identified they can be used as part of a strategy to vaccinate against specific

5 TAAs (therapeutic applications).



-99-

#### Fourth Series of Experiments

The selective production of monoclonal antibodies (MAbs) reacting with defined cell surface expressed molecules is now readily accomplished with an immunological subtraction approach, surface-epitope masking (SEM). Using SEM, prostate carcinoma (Pro 1.5) MAbs have been developed that react with tumor associated antigens expressed on human prostate cancer cell lines and patient-derived carcinomas. Screening a human LNCaP prostate cancer cDNA expression library with the Pro 1.5 MAb identifies a gene, prostate carcinoma tumor antigen-1 (PCTA-1). PCTA-1 encodes a secreted protein of ~35 kDa that shares ~40% sequence homology with the N-amino terminal region of members of the S-(soluble) galactose-binding lectin (galectin) gene family. Specific galectins are found on the surface of human and murine neoplastic cells and have been implicated in tumorigenesis and metastasis. Primer pairs within the 3' untranslated region of PCTA-1 and reverse transcription-PCR demonstrate selective expression of PCTA-1 by prostate carcinomas versus normal prostate and benign prostatic hypertrophy. These findings document the use of the SEM procedure for generating MAbs reacting with tumor associated antigens expressed on human prostate cancers. The SEM-derived MAbs have been used to expression clone the gene encoding this human tumor antigen. The approaches described, SEM combined with expression cloning, should prove of wide utility for developing immunological reagents specific for and identifying genes relevant to human cancer.

Production of MAbs reacting with antigens present on the surface of tumor cells, but displaying restricted expression on normal cells, is often a difficult and

-100-

inefficient process (1-3). A procedure based on immunological subtraction, SEM, has been developed that in principle can obviate many of the limitations preventing efficient MAb development toward molecules expressed on the cell surface (3,4). SEM is based on the selective blocking of antigens on a genetically modified target cell, i.e., tester, with polyclonal antibodies produced against the same unmodified cell line, i.e., driver. Antigen blocked cells are injected into BALB/c mice and sensitized spleen cells are removed, fused to NS1 myeloma cells and hybridomas secreting reactive MAbs are isolated (3). The SEM approach has been successfully used for a number of applications resulting in the production of MAbs specific for surface expressed molecules with known and unknown functions (3,4). These include MAbs reacting with the 170,000 M<sub>r</sub> human multidrug resistance protein (P-glycoprotein) and the human interferon gamma receptor (3,4). In addition, SEM has been used to produce MAbs, Pro 1.1 to 1.5, reacting with TAAs expressed on appropriate genetically modified CREF-Trans 6 and human prostate carcinoma cell lines (3).

An improved procedure has been developed for identifying and cloning dominant acting oncogenes, termed rapid expression cloning (5,6). This approach involves transfecting high molecular weight human tumor DNA into a new acceptor cell line, CREF-Trans 6, selecting cells expressing genes inducing tumors in nude mice and using molecular biological approaches, such as differential RNA display, to clone the putative oncogene (5,6). Tumor-derived CREF-Trans 6 cells have also proven useful for identifying TAAs expressed on the cell surface of cancer cells serving as the initial source for transforming DNA (5,6). Expression cloning of cDNAs using antibodies reacting with their encoded proteins

-101-

represents a direct means of identifying and cloning functional genes (2). This approach has now been used to identify and clone a gene, prostate carcinoma tumor antigen-1 (PCTA-1), encoding TAAs recognized by the MAb Pro 1.5. Sequence analysis indicates that PCTA-1 consists of a cDNA of 3.8 Kb with no homology to previously identified genes. Some overlapping homologies are detected with several small, noncontiguous partial cDNA sequences (of less than 500 nucleotides) previously identified as expressed human sequence tags (7). Analysis of protein structure indicates that PCTA-1 has ~40% homology to specific structural domains of the S-lectin family of galactose-binding lectin proteins, the galectins (8-10). These highly homologous proteins include a carbohydrate-binding 35 kDa protein CBP35 expressed on NIH-3T3 fibroblasts, a 34 kDa galactose-binding surface antigen present on metastatic murine tumors, a 31 kDa galactose-binding surface protein on metastatic human tumors, a 30 kDa carbohydrate-binding protein CBP30 found on baby hamster kidney cells, rat and human lung 29 kDa galactose-binding lectins, an IgE-binding protein of rat basophilic cells and a 32 kDa surface antigen Mac-2 found on thioglycollate-elicited murine macrophages (6-8). The roles of the S-lectin (galectin) proteins are diverse and impinge upon important biological processes including cell signaling, proliferative control, cell adhesion and cell migration (8-10). In this context, the demonstration that PCTA-1 encodes a protein with homology to the S-lectins (galectins) places this molecule in a pivotal position relative to human prostate cancer development and evolution.

-102-

**Materials and Methods****Cell lines**

The LNCaP cell line was derived from metastatic deposits from a patient with advanced prostate cancer (11).  
5 CREF-Trans 6 and LNCaP DNA-transfected nude mouse tumor-derived CREF-Trans 6 cells, CREF-Trans 6:4 NMT, were isolated as described previously (5). The hormone independent prostatic carcinoma cell lines DU-145 and PC-3 were obtained from the American Type Culture  
10 Collection. Conditions for growing the various cell types were as described previously (5,6,11).

**cDNA library construction, expression cloning and sequencing**

15 An LNCaP cDNA library was constructed in the Uni-ZAP XR vector (Stratagene®) (6,12,13). The cDNA library was screened using the Pro 1.5 MAb following the protocol in the picoBlue Immunoscreening Kit (Stratagene). Host bacterial cells (SURE) were plated on fifteen 150-mm X  
20 15-mm NZY plates to yield ~20,000 plaques/plate. After 3.5 hr incubation at 42°C, nitrocellulose filters soaked in 10 mM IPTG solution were added to the top of colonies for plaque lifts. The filters were washed 3 to 4X with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20)  
25 and soaked in blocking solution [1% BSA in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl)] and incubated for 1 hr at room temperature. The filters were then transferred into fresh blocking solution containing Pro 1.5 ascites (1:500 dilution) and incubated for 3 hr at room temperature with  
30 gentle agitation. After washing 4X with TBST buffer, the filters were transferred into fresh blocking solution containing Ab-AP conjugate (1:2000 dilution) and incubated for 1 hr at room temperature. Positive colonies were identified by developing the filters in a solution  
35 containing 0.3 mg/ml NBT (nitroblue tetrazolium), 0.15

-103-

mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate), 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM MgCl<sub>2</sub>. The reaction was terminated by adding stop solution (20 mM Tris-HCl pH 2.9 and 1 mM EDTA. Positive 1 clones  
5 containing PCTA-1 were isolated. The complete sequence of PCTA-1 was obtained using the Sanger method (14). A series of oligonucleotides synthesized from both sides of the PCTA-1 insert in the pBluescript vector were used as primers. Sequence analysis was verified using an Applied  
10 Biosystems (Model 373A, Version 1.2.1) sequencer .

#### **In vitro translation of PCTA-1**

The plasmid DNA containing PCTA-1 was linearized by digestion with Xma III and used as a template to  
15 synthesize mRNA using the mCAP mRNA capping kit (Stratagene). In vitro translation of PTI-1 was performed using a rabbit reticulocyte lysate translation kit with conditions as described by GibcoBRL (MD) (6).

#### **20 Preparation of mouse polyclonal antibodies and SEM**

CREF-Trans 6 polyclonal antibodies were prepared as described (3). The CREF-Trans 6 polyclonal antibodies were used to coat CREF-Trans 6:4 NMT cells by the SEM approach resulting in the production of hybridomas  
25 secreting Pro 1.5 MAbs (3).

#### **Fluorescence cell staining and immunostaining of tissue sections with Pro 1.5 and PSA**

Fluorescence staining using Pro 1.5 MAb used previously  
30 described protocols (15). Tissue sections were prepared from fresh tissues frozen in liquid nitrogen. Serial sections for several tissues were used to prepare RNA for RT-PCR (6). Staining of tissue sections used standard protocols (Super Sensitive Detection System (BioGenex).  
35 Briefly, sections were fixed in acetone, blocked with 3

-104-

% H<sub>2</sub>O<sub>2</sub> for 7 min at room temperature and incubated for 20 min at room temperature in PBS containing 3 % lamb serum. Sections were then incubated with Pro 1.5 (1:100) or PSA (1:200) (DAKO Corporation) for 45 min at room temperature. Samples were incubated with biotinylated secondary antibody and alkaline phosphatase conjugated streptavidin in PBS. Prior to each incubation step, sections were washed 3 to 5X with PBS. Reactivity was detected by adding DAB and counterstaining with Hematoxylin.

#### Immunoprecipitation analysis

Cells were labeled with <sup>35</sup>S-methionine and cell lysates were analyzed for PCTA-1 protein levels by immunoprecipitation analysis with Pro 1.5 MAbs as previously described (3,16). Secreted PCTA-1 was detected by labeling cells for 4 hr with <sup>35</sup>S-methionine, growing cells for an additional 24 hr in the absence of label, collecting the medium, concentrating the medium and performing immunoprecipitation analysis with Pro 1.5 MAbs.

#### RNA preparation and RT-PCR

Total cytoplasmic RNA was isolated from logarithmically growing cell cultures as previously described (6,13). Tissue samples from normal prostates and patients with prostatic carcinomas or BPH were frozen in liquid nitrogen and RNA was isolated using the TRIzol reagent as described by GibcoBRL (MD). Tissue samples were supplied by the Cooperative Human Tumor Network (CHTN). Three samples of normal prostate were obtained from autopsies of males < 40 years of age. All tissues were histologically confirmed as normal, BPH or carcinoma of the prostate (6). RT-PCR using primer pairs for PCTA-1, PSA and GAPDH was performed as described previously

-105-

(6,16).

### Experimental Results

- 5      Production of Pro 1.5 MAbs by SEM and reactivity of Pro 1.5 and PSA with normal prostate, BPH and prostate carcinomas

10      The SEM approach of blocking antigenic epitopes on nude mouse tumor-derived LNCaP DNA transfected CREF-Trans 6 cells with CREF-Trans 6 polyclonal antibody prior to injection into mice was used to produce hybridomas secreting the Pro (prostate carcinoma) series of MAbs (3). The Pro MAbs can detect by in situ fluorescence

15      microscopy and fluorescence activated cell sorter (FACS) analysis the surface expression of tumor associated antigens on LNCaP-transfected primary (CREF-Trans 6:4 NMT) and secondary tumor-derived CREF-Trans 6 cells and LNCaP, DU-145 and PC-3 human prostate cancer cell lines

20      (3) (Fig. 16A, 16B, 16C and data not shown). In contrast, Pro 1.5 does not react using fluorescence microscopy or FACS with CREF-Trans 6 cells (3). The staining pattern with Pro 1.5 in human prostate carcinoma cells is irregular with microclusters, as previously observed with

25      MAbs reacting with specific galectins (10,17,18) (Figs. 16A, 16B, 16C). Immunoprecipitation analysis identifies an ~35 to 42 kDa protein in lysates from LNCaP-transfected primary and secondary tumor-derived CREF-Trans 6, LNCaP, DU-145 and PC-3 cells (3) (Fig. 1D

30      and data not shown). Of the prostate cancer cell lines studied, the PC-3 clone contains the lowest quantities of cell associated, surface expressed (FACS) and secreted PCTA-1 protein.

- 35      To determine if Pro 1.5 MAbs can react with

-106-

patient-derived prostate cancer specimens, frozen sections were prepared from normal (males < 40 years of age), BPH and carcinomas of the prostate. Normal prostate displays limited reactivity with Pro 1.5, whereas PSA readily stains normal prostate epithelial cells (Fig. 17). As expected, PSA also stains prostate cells in tissue sections of BPH and prostate carcinomas. MAb Pro 1.5 reacts strongly with prostate carcinoma cells present in frozen tissue sections, but not with adjacent benign glands or tissue sections containing normal prostate or BPH epithelium. However, some reactivity with Pro 1.5 is found in prostatic intraepithelial neoplasia (PIN). These studies indicate that the Pro 1.5 MAb can distinguish between prostate carcinoma and PIN versus normal prostate epithelial cells and BPH. In this context, Pro 1.5 provides a discriminatory capacity for detection of cancer of the prostate that exceeds that of the nonspecific prostate epithelial cell marker PSA.

**20 Expression cloning of PCTA-1 using SEM-derived Pro 1.5 MAbs.**

To identify the gene encoding the TAAs identified on human prostate cancer cells by MAb Pro 1.5 an antibody expression cloning strategy was used. An LNCaP cDNA library was constructed in the Uni-ZAP XR vector (Stratagene®) and screened with the Pro 1.5 MAb (12,13). This approach resulted in the identification of a 3.8 Kb cDNA clone referred to as PCTA-1. In vitro protein translation of the PCTA-1 cDNA results in a 317 aa protein of ~35 kDa (data not shown). Although the DNA sequence of PCTA-1 displays no homology to previously identified genes, protein comparison indicates that the PCTA-1 protein is ~40% homologous to specific regions of the S-lectin family of galactose-binding lectin proteins,



-107-

the galectins (Figs. 18A, 18B). Several types of protein sequence homologies are found between PCTA-1 and the other galectins. Stretches of identical amino acids are found in PCTA-1 and both the small ~14 kDa and larger ~29 to 34 kDa galectins, e.g., HFNPRF, IVCN and WG. These amino acids are well conserved and are found in galectins isolated from diverse species, including eel, chicken, mouse, rat, bovine and human (Fig. 18B) (10,19,20). They are important structural components of the galectins and may mediate galectin binding to its putative ligands (10). Of potential interest is the replacement in PCTA-1 of two amino acids normally present in the conserved regions of most of the galectins, i.e., the substitution of an I for an R and a T for an F (Fig. 18B). A number of similar positioned amino acids are found in PCTA-1 and the larger ~29 to 34 kDa human and mouse galectins, that are not present in the ~14 kDa galectins isolated from most other species, e.g., DVAF and WGREE (Fig. 18B). In addition, a number of amino acids are distinct for PCTA-1 versus the other similar positioned amino acids that are found in the human galectin-3 L29 and Human L-31 proteins, including RA, KRAG, LI, and ITYDT. The similarities in regions of conserved structure between PCTA-1 and the galectins suggest that they will share a number of overlapping properties. However, the numerous amino acid changes observed in PCTA-1, in both the conserved regions and throughout the remainder of this protein, can be expected to impact on the structure and affect the properties of the PCTA-1 protein.

30

#### Expression of PCTA-1 in cell lines and normal prostate, BPH and prostate carcinomas

35

After obtaining the sequence of PCTA-1, studies were performed to determine if primers for specific regions of

-108-

this gene could be identified that would permit detection of RNA expression by RT-PCR. Using primers located between bp 3010 and bp 3423 in the 3' untranslated region, PCTA-1 expression is apparent in LNCaP DNA-transfected tumor-derived CREF-Trans 6, LNCaP and DU-145 cells, but not in untransfected CREF-Trans 6 cells (Fig. 19). PCTA-1 expression also occurs in seven of seven patient-derived prostate carcinomas, one of four BPH and one of four putative normal prostate tissue samples (Fig. 19). In the one BPH sample displaying PCTA-1 expression, histological analysis indicated the presence of epithelial atypia consistent with PIN. In the one putative normal prostate tissue sample expressing PCTA-1, no tissue was available for histological analysis. However, since this tissue was obtained from a 60 year-old male, it is possible that this tissue may have contained clinically unsuspected prostate disease. The same RNA samples were tested for prostate specific antigen (PSA) expression (Fig. 19). PSA RNA was present in LNCaP cells and all of the prostate tissue samples, but not in CREF-Trans 6, LNCaP DNA-transfected tumor-derived CREF-Trans 6 or DU-145 cells. As predicted, all samples were positive for expression of the housekeeping gene GAPDH (Fig. 19). Although the sampling size is small, these results indicate that PCTA-1 expression may be restricted to prostate carcinomas and subsets of BPH displaying early stages of cancer, i.e., PIN. In contrast, PCTA-1 expression is not evident in normal prostate or BPH.

30 Secretion of PCTA-1 by human prostate carcinoma cell lines. Many galectins are externalized by non-classical secretory mechanisms that do not involve a typical secretion signal peptide (10,17,18). In order to  
35 determine if PCTA-1 is secreted by Pro 1.5 MAb positive

-109-

cells, target cells were labeled for 4 hr with  $^{35}\text{S}$ -methionine, the label was removed and cells were washed three times in methionine-free medium and then incubated for an additional 18 to 24 hr in complete medium without label. The conditioned medium (CM) was collected, contaminating cells were removed, the CM was concentrated and immunoprecipitated using the Pro 1.5 MAb (Fig. 16C). A protein of ~35 to 42 kDa was present in CM from CREF-Trans 6:4 NMT, LNCaP, DU-145 and PC-3, but not from CM obtained from CREF-Trans 6 cells (Fig. 16C). Immunoprecipitation analysis of  $^{35}\text{S}$ -methionine labeled cell lysates from the same cell types indicate a similar pattern of PCTA-1 expression, i.e., present in CREF-Trans 6:4 NMT and human prostate carcinoma cell lines, but not in untransfected CREF-Trans 6 cells (Fig. 16C). As previously found using FACS analysis with the Pro MABs, PC-3 cells produced the lowest levels of cell-associated and secreted PCTA-1. These results demonstrate that PCTA-1 is shed from prostate cancer cells. In this context, monitoring PCTA-1 protein in the circulation might prove beneficial as a diagnostic marker for prostate cancer.

#### Experimental Discussion

The early detection of prostate cancer and the accurate prediction of its clinical course is not possible using current methodologies. Contemporary approaches, including physical examination, tissue biopsy, monitoring serum PSA levels, and ultrasound and bone scans do not insure early prostate cancer detection and are of only limited value in predicting disease progression. Of immense value for the accurate diagnosis and potentially for the therapy of human prostate cancer is the identification of immunological and genetic reagents displaying the

-110-

appropriate specificity that will permit a clear distinction between prostate carcinoma versus normal prostate and BPH. By using a number of innovative strategies, including rapid expression cloning, SEM and antibody expression cloning, we have produced MABs reacting with TAAs differentially expressed on human prostate carcinoma cells versus normal prostate and BPH and have cloned the gene PCTA-1 that encodes this protein. PCTA-1 is expressed in invasive prostate carcinoma and early prostate cancer, PIN, but not in histologically confirmed normal prostate or BPH. The PCTA-1 encoded TAAs are detected on the surface of prostate cancers and are shed by prostate carcinoma cells. These attributes should allow the direct use of the Pro series of MABs and the PCTA-1 gene for diagnostic applications. If appropriate specificity of the PCTA-1 gene is found using a larger tissue sampling, this gene may also prove useful for designing gene-based strategies for the therapy of prostate cancer.

The PCTA-1 protein retains a number of conserved structural motifs that are found in most members of the galectin gene family (10,19,20). These conserved regions are present in species as diverse as eel, mouse, rat and human (10). On the basis of the DNA sequence of PCTA-1 and its encoded protein, PCTA-1 is a new member of the galectin gene family, galectin-8, that may contribute to the cancer phenotype of human prostate carcinomas. The galectins display wide tissue distribution, clear developmental regulation and differential levels in specific tissues, supporting the hypothesis that they contribute to many physiologically important processes in mammalian cells (10). Of direct relevance to cancer, is the finding that the galectins, as well as the selectin subgroup of C-type lectins (21,22), can mediate both

-111-

cell-cell and cell-matrix interactions (10,17,23). These associations are critical elements in mediating the metastatic spread of tumor cells (24). Moreover, experimental evidence has accumulated indicating that

5 galectin-3 may play an important role in the metastatic process (17,25-29). Galectin-3 is overexpressed in human colon and gastric carcinomas versus normal and benign tissue and elevated expression of recombinant L-34 in a

10 weakly metastatic UV-2237-cl-15 mouse fibrosarcoma cell line increases lung metastases in syngeneic and nude mice (17,25-29). Moreover, anti-galectin MABs inhibit homotypic aggregation, anchorage-independent growth and experimental metastases in UV-2237 subclones (30-32). Studies are in progress to determine if the PCTA-1 gene

15 and the Pro MABs display similar properties as the cloned galectin-3 gene and the anti-galectin MABs, respectively. It will also be important to ascertain if inhibition in PCTA-1 expression, using antisense oligonucleotides, antisense expression vectors or ribozyme approaches,

20 alters the tumorigenic or metastatic properties of human prostate cancers.

Prior to SEM, no simple and direct procedure was available for efficiently generating MABs reacting with

25 differentially expressed surface molecules. Conceptually, SEM involves immunological subtraction that induces the immune system of mice to target antibody production toward surface molecules expressed on a genetically modified tester cell line, but not expressed or expressed

30 in lower abundance on its' cognate unmodified driver cell line (3,4). By using polyclonal antibodies produced against the driver cell line to coat (mask) epitopes on the tester cell line, enriched production of MABs targeted toward epitopes expressed on the surface of the

35 tester cell type is achieved (3,4). In addition to

-112-

identifying TAAs present on the surface of human prostate cancer cells, Pro 1.1 to 1.5, the SEM approach has also been used with rapid expression cloning to develop MAb's reacting with human breast carcinoma cells (data not shown). These results indicate that these approaches may represent an efficient strategy for identifying antigenic epitopes on human cancers. Additional applications of SEM may also result in the targeted production of MAb's and the identification of genes associated with important physiological processes, including cellular growth and differentiation, immunological recognition, tumorigenesis, metastasis, cellular senescence, atypical multiple drug resistance and autoimmune disease.

In summary, applicants presently demonstrate direct applications of the rapid expression cloning and SEM technologies for the development of MAb's and the cloning of the PCTA-1 gene associated with human prostate cancer. The PCTA-1 gene and the recently identified prostate tumor inducing gene PTI-1 (6) are genetic elements that can distinguish prostate cancer from normal prostate and BPH. Although further studies are required, it appears that PCTA-1 may represent an earlier genetic change in human prostate cancer development than PTI-1. In this context, both the Pro MAb's and the PCTA-1 gene should find direct applications for prostate cancer diagnosis and staging and they may also represent important therapeutic reagents for intervention in this pervasive and often fatal neoplastic disease.

-113-

References of the Fourth Series of Experiments

1. Waldmann, T.A. (1991) Science 252, 1657-1662.
2. Leon, J.A., Goldstein, N.I. & Fisher, P.B. (1994)  
5 Pharmacol. Therapeut.. 61, 237-278.
3. Shen, R., Su, Z.-z., Olsson, C.A., Goldstein, N.I.  
& Fisher, P.B. (1994) J. Natl. Cancer Inst. 86,  
91-98.
4. Fisher, P.B. (1995) Pharmaceutical Tech. 19, 42-48.
- 10 5. Su, Z.-z., Olsson, C.A., Zimmer, S.G. & Fisher, P.B.  
(1992) Anticancer Res. 12, 297-304.
6. Shen, R., Su, Z.-z., Olsson, C.A. & Fisher, P.B.  
(1995) Proc. Natl. Acad. Sci. USA 92, 6778-6782.
7. Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno,  
15 R., Kelley, J. M., Utterback, T. R., Nagle, J. W.,  
Fields, C. & Venter, J. C. (1992) Nature (London)  
355, 632-634.
8. Hughes, R. C. (1992) Curr. Opin Struct. Biol. 2,  
687-692.
- 20 9. Hirabayashi, J. & Kasai, K.-i. (1993) Glycobiology  
4, 297-304.
10. Barondes, S.H., Cooper, D.N.W., Gitt, M.A. &  
Leffler, H. (1994) J. Biol. Chem. 269, 20807-20810.
11. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr,  
25 J.P., Rosenthal, H., Ming Chu, T., Mirand, E.A. &  
Murphy, G.G. (1983) Cancer Res. 43, 1809-1818.
12. Reddy, P.G., Su, Z.-z. & Fisher, P.B. (1993) in  
Chromosome and Genetic Analysis, Methods in  
Molecular Genetics, ed. Adolph, K. W., (Academic  
30 Press, Inc., Orlando, FL), vol. 1, pp. 68-102.
13. Jiang, H. & Fisher, P.B. (1993) Mol. Cell.  
Different. 1, 285-299.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977)  
Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- 35 15. Miranda, A., Duigou, G. J., Hernandez, E. & Fisher,

-114-

- P. B. (1988) J. Cell Sci. 89, 481-493.
16. Jiang, H., Lin, J., Su, Z.-z., Herlyn, M., Kerbel, R.S., Weissman, B.E., Welch, D.R. & Fisher, P.B. (1995) Oncogene, 10, 1855-1864, 1995.
- 5 17. Raz, A. & Lotan, R. (1987) Cancer Metastasis Rev., 6, 433-452.
18. Lindstedt, R., Apodaca, G., Barondes, S.H., Mostov, K.E. & Leffler, H. (1993) J. Biol. Chem., 268, 11750-11757.
- 10 19. Raz, A., Carmi, P. & Pazerini, G. (1987) Cancer Res. 48, 645-649.
20. Raz, A., Carmi, P., Raz, T., Hogan, V., Mohamed, A. & Wolman, S. R. (1991) Cancer Res. 51, 2173-2178.
21. Drickamer, K. & Taylor, M. E. (1993) Annu. Rev. Cell Biol. 9, 237-264.
- 15 22. Rosen, S. D. (1993) Semin. Immunol. 5, 237-247.
23. Barondes, S. H. (1984) Science 223, 1259-1264.
24. Liotta, L. A., Steeg, P. G. & Stetler-Stevenson, W. G. (1991) Cell 64, 327-336.
- 20 25. Raz, A., Zhu, D., Hogan, V., Shah, N., Raz, T., Karkash, R., Pazerini, G. & Carmi, P. (1990) Int. J. Cancer 46, 871-877.
26. Irimura, T., Matsushita, Y., Sutton, R. C., Carralero, D., Ohannesian, D. W., Cleary, K. R., Ota, D. M., Nicolson, G. L. & Lotan, R. (1991). Cancer Res. 51, 387-393.
- 25 27. Lotan, R., Ito, H., Yasui, W., Yokoaki, H., Lotan, D. & Tahara, E. (1994) Int. J. Cancer 56, 474-480.
28. Schoeppner, H. L., Raz, A., Ho, S. B. & Bresalier, R. S. (1995) Cancer 75, 2818-2826.
- 30 29. Ohannesian, D. W., Lotan, D., Jessup, P. T. J. M., Fukuda, M., Gabius, H.-J. & Lotan, R. (1995) Cancer Res. 55, 2191-2199.
- 30 30. Lotan, R., Lotan, D. & Raz, A. (1985) Cancer Res. 45, 4349-4353.



-115-

31. Raz, A., Meromsky, L. & Lotan, R. (1986) Cancer Res. 46,3667-3672.
32. Meromsky, L., Lotan, R. & Raz, A. (1986) Cancer Res. 46,5270-5276.

-116-

Fifth Series of Experiments

Rapid expression cloning and differential RNA display identified a novel prostatic carcinoma oncogene, prostate tumor inducing gene-1 (PTI-1). PTI-1 consists of a 630 bp 5'-UTR with sequence homology to *Mycoplasma hyopneumoniae* (5'-UTR) and a 3' sequence encoding a truncated and mutated form of human elongation factor-1a. Screening a cDNA library constructed from LNCaP human prostate cancer cells with the 5'-UTR identifies additional PTI cDNAs with similar 5'-UTR regions, but differing 3' regions. Southern hybridization analysis of genomic DNAs demonstrates the presence of PTI 5'-UTR sequences in organisms as diverse as bacteria, yeast and human. Using a 5'-UTR probe of PTI-1, RNA expression occurs in cloned rat embryo fibroblast (CREF) cells transformed by diverse acting viral oncogenes, including adenovirus type 5 (Ad5), a cold-sensitive host-range Ad5 mutant, T24 Ha-ras, v-src and human papilloma virus type-51 (HPV-51). A CREF clone containing a wild-type Ad5 E1A transforming gene under the transcriptional control of a dexamethasone (DEX)-inducible mouse mammary tumor virus promoter demonstrates a direct relationship between induction of Ad5 E1A, the 5'-UTR of PTI-1 and cellular transformation. Expression of the 5'-UTR of PTI-1 is regulated at a transcriptional level in the DEX-inducible Ad5 E1A-transformed CREF clone, as well as in v-src, Ha-ras and HPV-51-transformed CREF cells. These results indicate that morphological and oncogenic transformation of CREF cells correlates with transcriptional induction of the 5'-UTR of PTI genes. Since this 5'-UTR sequence is a component of multiple cellular RNAs, genes linked to this 5'-UTR may be targets for oncogenic transformation.

It is now possible to detect dominant-acting

-117-

tumor-inducing oncogenes in genomic DNAs from human cancer cell lines and primary patient-derived tumors using the rapid expression cloning procedure with a new DNA acceptor cell line CREF-Trans 6. This approach has

5 been used with high molecular weight DNA from the human LNCaP prostate carcinoma cell line to demonstrate the presence of a putative nude mouse tumor inducing gene, not detected using similar approaches with NIH-3T3 cells. Tumors developing in nude mice injected with LNCaP DNA

10 transfected CREF-Trans 6 cells were established in culture, mRNAs were compared by differential RNA display (DD) and a novel sequence of 214 bp (representing part of the 5'-UTR of PTI-1) was identified and cloned. When Northern blots containing RNAs from various cell types

15 were probed with the 214 bp sequence, RNA expression was detected in LNCaP transfected nude mouse tumor-derived CREF-Trans 6, LNCaP, DU-145 (hormone refractile human prostate carcinoma), NCI-H69 (human small cell lung carcinoma), T47D (human breast carcinoma), SW-480 and

20 LS174T (human colorectal carcinomas) cells. Screening an LNCaP cDNA library with the 214-bp DNA fragment and RACE 5' extension resulted in the cloning of a full-length 2.0-kb PTI-1 cDNA. Sequence analysis indicates that PTI-1 consists of a 630-bp 5' sequence with homology to

25 *Mycoplasma hyopneumoniae* and a 3' sequence homologous to a truncated and mutated form of human elongation factor 1-a (EF-1a). Using a pair of primers recognizing a 280-bp region within the 630-bp 5'-UTR PTI-1 sequence, reverse transcription-PCR detects PTI-1 expression in

30 patient-derived prostate carcinomas but not in normal prostate or benign hypertrophic prostate tissue. These findings indicate that PTI-1 may be a member of a class of oncogenes that could affect protein translation and contribute to carcinoma development in human prostate and

35 other tissues.

-118-

The present study was designed to determine the potential significance of the mycoplasmal-like 5'-UTR region of PTI-1. We demonstrate that this sequence is present in genomic DNA of both procaryotic and eukaryotic cells. In humans, tissue expression of the 5'-UTR occurs in normal skeletal muscle and colon tissue, as well as in specific carcinomas of the prostate, colon, lung and breast. cDNAs isolated from an LNCaP cDNA library indicate that the 5'-UTR is associated with multiple distinct 3' regions, suggesting that this sequence is part of a PTI multigene family. Expression of the 5'-UTR of PTI-1 correlates with transformation in rat embryo fibroblast cells by diverse DNA viruses. The induction of the 5'-UTR in oncogene-transformed rat embryo cells occurs by a transcriptional mechanism. These observations suggest that cDNAs containing the 5'-UTR of PTI-1 may be targets for transcriptional activation occurring during oncogenic transformation.

-119-

**What is claimed is:**

1. An isolated mammalian nucleic acid molecule encoding  
the Prostate Carcinoma Tumor Antigen Gene-1  
protein.  
5
2. A DNA molecule of claim 1.
3. A genomic DNA molecule of claim 2.  
10
4. A cDNA molecule of claim 2.
5. An RNA molecule of claim 1.
- 15 6. An isolated human nucleic acid molecule of claim 1.
7. A nucleic acid molecule of at least 15 nucleotides  
capable of specifically hybridizing with a unique  
sequence of the nucleic acid molecule of claim 1.  
20
8. An antisense oligonucleotide having a sequence  
capable of specifically hybridizing to an RNA  
molecule encoding the Prostate Carcinoma Tumor  
Antigen Gene-1 protein so as to prevent translation  
or cause degradation of the RNA molecule.  
25
9. A method of detecting expression of the Prostate  
Carcinoma Tumor Antigen Gene-1 in a sample which  
contains cells comprising steps of:  
30
  - (a) obtaining total RNA from the cells;
  - (b) contacting the RNA so obtained with a labelled  
nucleic acid molecule of claim 7 under  
hybridizing conditions; and
  - (c) determining the presence of RNA hybridized to  
35 the molecule, thereby detecting the expression

-120-

of the Prostate Carcinoma Tumor Antigen Gene-1  
in the sample.

- 5 10. A method of detecting expression of the Prostate Carcinoma Tumor Antigen Gene-1 in tissue sections which comprises steps of:
  - 10 (a) contacting the tissue sections with a labelled nucleic acid molecule of claim 7 under hybridizing conditions permitting hybridization of the molecule of claim 7 and the RNA of Prostate Carcinoma Tumor Antigen Gene-1; and
  - 15 (b) determining the presence of RNA hybridized to the molecule of claim 7, thereby detecting the expression of the Prostate Carcinoma Tumor Antigen Gene-1 in tissue sections.
- 20 11. An isolated mammalian nucleic acid molecule of claim 2 operatively linked to a promoter of RNA transcription.
- 25 12. A vector which comprises the isolated mammalian nucleic acid molecule of claim 1.
13. A plasmid of claim 12.
14. A virus of claim 12.
15. A DNA virus of claim 14.
- 30 16. An RNA virus of claim 14.
17. A retrovirus of claim 14.
- 35 18. The plasmid of claim 13 designated PCTA-1, having ATCC Accession No. 97021.

-121-

19. A host vector system for the production of a polypeptide having the biological activity of a mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein which comprises the vector of claim 12 and a suitable host.
20. A host vector system of claim 19, wherein the suitable host is selected from a group consisting of a bacterial cell, an insect cell, a plant cell and a mammalian cell.
21. A method of producing a polypeptide having the biological activity of a mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein which comprises growing the host cells of the host vector system of claim 16 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
22. A method of transforming cells which comprises introducing a vector of claim 12 into an appropriate host cell.
23. Transformed cells of claim 22.
24. Purified mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein.
25. A polypeptide encoded by the isolated mammalian nucleic acid molecule of claim 1.
26. An antibody capable of specifically binding to the mammalian Prostate Tumor Inducing Gene-1 protein of claims 20 or 21.

-122-

27. A monoclonal antibody of claim 26.
28. A compound capable of competitively inhibiting the binding of the antibody of claim 26 or 27 with the mammalian Prostate Tumor Inducing Gene-1 protein.
29. A pharmaceutical composition comprising the antibody of claim 26 or 27 or the compound of claim 28 and a pharmaceutically acceptable carrier.
30. A therapeutic agent comprising an antibody of claim 26 or 27 and a cytotoxic agent.
31. A therapeutic agent of claim 30, wherein the cytotoxic agent is either a radioisotope or toxin.
32. A method for measuring the amount of the mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein in a biological sample comprising steps of:
- (a) contacting the sample with the antibody of claim 26 or 27 under conditions permitting formation of a complex between said antibody and the mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein, and
  - (b) measuring the amount of said complex, thereby measuring the amount of the Prostate Carcinoma Tumor Antigen Gene-1 protein in said biological sample.
33. A method for measuring the amount of the mammalian Prostrate Carcinoma Tumor Antigen Gene-1 protein in a biological sample comprising steps of:
- (a) contacting the sample with a capturing monoclonal antibody capable of binding to the mammalian Prostrate Carcinoma Tumor Antigen



-123-

- Gene-1 protein and is bound on a solid matrix under conditions permitting formation of a complex between the mammalian Prostrate Carcinoma Tumor Antigen Gene-1 protein and the bound antibody;
- 5 (b) removing the unbound sample;
- (c) contacting the solid matrix with a secondary antibody which is capable of binding to the mammalian Prostrate Carcinoma Tumor Antigen
- 10 Gene-1 protein in at least one site other than the capturing antibody; and
- (d) measuring the amount of said complex, thereby measuring the amount of the Prostate Carcinoma Tumor Antigen Gene-1 protein in said biological
- 15 sample.
34. A method of claim 33, further comprising before step d, contacting a tertiary antibody which is labelled with a detectable marker and capable of binding the
- 20 secondary antibody.
35. A method of claim 32, 33 or 34 wherein the sample is a serum sample.
- 25 36. A method to determining whether a subject carries a cancer with metastatic potential comprising steps of:
- (a) measuring the amount of Prostate Carcinoma Tumor Antigen Gene-1 protein in the serum
- 30 sample of the subject; and
- (b) comparing the amount determined in step (a) with the amount determined from the sample of a healthy subject or a subject with benign tumor.

35

-124-

37. A method for determining whether a compound is capable of inhibiting the expression or activity of Prostate Carcinoma Tumor Antigen Gene-1 protein comprising steps of:
- 5 (a) contacting the transformed cells of claim 19 with an appropriate amount of the compound under conditions permitting the compound to inhibit expression or activity of Prostate Carcinoma Tumor Antigen Gene-1 protein; and
- 10 (b) detecting the level of the Prostate Carcinoma Tumor Antigen Gene-1 protein expression, a decrease in the expression level indicating that the compound is capable of inhibiting the expression or activity of Prostate Carcinoma
- 15 Tumor Antigen Gene-1 protein.
38. A method for determining whether a compound is capable of specifically binding to the Prostate Carcinoma Tumor Antigen Gene-1 protein comprising
- 20 steps of:
- (a) contacting an appropriate amount of the purified protein of claim 20 with an appropriate amount of the compound under conditions permitting formation of a complex
- 25 between the compound and the purified protein;
- (b) detecting such complex, the presence of the complex indicating that the compound is capable of binding to the Prostate Carcinoma Tumor
- 30 Antigen Gene-1 protein.
39. A pharmaceutical composition comprising an effective amount of the compound capable of either binding or inhibiting the activity of the Prostate Carcinoma Tumor Antigen Gene-1 protein as determined by method
- 35 of claim 31 or 32 and a pharmaceutical carrier.

-125-

- 5 40. A method of treating cancer with metastatic potential in a subject comprising administering effective amount of the pharmaceutical composition of claim 29, therapeutic agent of claim 30 or 31, the pharmaceutical composition of 39, alone or in combination of, to the subject.
- 10 41. An isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-1.
42. A DNA molecule of claim 41.
- 15 43. A genomic DNA molecule of claim 41.
44. A cDNA molecule of claim 41.
45. An RNA molecule of claim 41.
- 20 46. An isolated human nucleic acid molecule of claim 41.
47. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 41.
- 25 48. An antisense oligonucleotide having a sequence capable of specifically hybridizing to an RNA molecule encoding the Prostate Tumor Inducing Gene-1 protein so as to prevent translation of the RNA molecule.
- 30 49. An antisense oligonucleotide having a sequence capable of inactivating the expression of the 5'-UTR of Prostate Tumor Inducing Gene-1 sequence.

35

-126-

50. A method of detecting expression of the Prostate Tumor Inducing Gene-1 in a sample which contains cells which comprises steps of:
- 5 (a) obtaining total RNA from the cell, contacting the RNA so obtained with a labelled nucleic acid molecule of claim 47 under hybridizing conditions; and
- 10 (b) determining the presence of RNA hybridized to the molecule, thereby detecting the expression of the Prostate Tumor Inducing Gene-1 in the sample.
51. A method of detecting expression of the Prostate Tumor Inducing Gene-1 in tissue sections which comprises steps of
- 15 (a) contacting the tissue sections with a labelled nucleic acid molecule of claim 47 under hybridizing conditions; and
- 20 (b) determining the presence of RNA hybridized to the molecule, thereby detecting the expression of the Prostate Tumor Inducing Gene-1 in tissue sections.
52. An isolated mammalian nucleic acid molecule of claim 25 41 operatively linked to a promoter of RNA transcription.
53. A vector which comprises the isolated mammalian nucleic acid molecule of claim 41.
- 30 54. A plasmid of claim 53.
55. The plasmid of claim 53 designated PTI-1 having ATCC Accession No. 97020.
- 35

-127-

56. A virus of claim 54.
57. A DNA virus of claim 54.
- 5 58. An RNA virus of claim 54.
59. A retrovirus of claim 54.
- 10 60. A host vector system for the production of a polypeptide having the biological activity of the mammalian Prostate Tumor Inducing Gene-1 protein which comprises the vector of claim 53 and a suitable host.
- 15 61. A host vector system of claim 60, wherein the suitable host is a bacterial cell, insect cell, plant cell or mammalian cell.
- 20 62. A method of producing a polypeptide having the biological activity of a mammalian Prostate Tumor Inducing Gene-1 protein which comprises growing the host cells of the host vector system of claim 61 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
- 25 63. A mammalian cell comprising the vector of claim 53.
- 30 64. Purified mammalian Prostate Tumor Inducing Gene-1 protein.
65. A polypeptide encoded by the isolated mammalian nucleic acid molecule of claim 41.
- 35 66. A method to produce antibody using the mammalian

-128-

Prostate Tumor Inducing Gene-1 protein of claim 64 or 65.

- 5      67. An antibody capable of binding specifically to the mammalian Prostate Tumor Inducing Gene-1 protein of claim 64 or 65.
68. A monoclonal antibody of claim 67.
- 10     69. A pharmaceutical composition comprising an antibody of claim 66 or 67 and a pharmaceutically acceptable carrier.
- 15     70. A therapeutic agent comprising an antibody of claim 66 or 67 and a cytotoxic agent.
71. A therapeutic agent of claim 70, wherein the cytotoxic agent is either a radioisotope or toxin.
- 20     72. An immunoassay for measuring the amount of a mammalian Prostate Tumor Inducing Gene-1 protein in a biological sample comprising steps of:
- 25            a) contacting the biological sample with the antibody of claim 66 or 67 under the condition permitting the formation of a complex with said antibody and the mammalian Prostate Tumor Inducing Gene-1 protein, and
- 30            b) measuring the amount of said complex, thereby measuring the amount of the Prostate Tumor Inducing Gene-1 protein in said biological sample.
- 35     73. An isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-2.

-129-

74. A DNA molecule of claim 73.
75. A genomic DNA molecule of claim 73.
- 5 76. A cDNA molecule of claim 73.
77. An RNA molecule of claim 63.
78. An isolated human nucleic acid molecule of claim 73.
- 10 79. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of the nucleic acid molecule of claim 73.
- 15 80. An antisense oligonucleotide having a sequence capable of inactivating the expression of the 5'-UTR of the Prostate Tumor Inducing Gene-2 protein sequence.
- 20 81. An isolated mammalian nucleic acid molecule of claim 73 operatively linked to a promoter of RNA transcription.
- 25 82. A vector which comprises the isolated mammalian nucleic acid molecule of claim 73.
83. A plasmid of claim 82.
- 30 84. The plasmid of claim 83 designated PTI-2 having ATCC Accession No. 69742.
85. A virus of claim 82.
- 35 86. A DNA virus of claim 82.

-130-

87. An RNA virus of claim 82.
88. A retrovirus of claim 82.
- 5 89. An isolated mammalian nucleic acid molecule having the sequence of Prostate Tumor Inducing Gene-3.
90. A DNA molecule of claim 89.
- 10 91. A genomic DNA molecule of claim 89.
92. A cDNA molecule of claim 89.
93. An RNA molecule of claim 89.
- 15 94. An isolated human nucleic acid molecule of claim 89.
95. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of the nucleic acid molecule of claim 86.
- 20 96. An antisense oligonucleotide having a sequence capable of specifically hybridizing to an RNA molecule encoding the Prostate Tumor Inducing Gene-3 protein so as to prevent translation or cause degradation of the RNA molecule.
- 25 97. An antisense oligonucleotide having a sequence capable of inactivating the expression of the 5'-UTR of the Prostate Tumor Inducing Gene-3 protein sequence.
- 30 98. A method of detecting expression of a Prostate Tumor Inducing Gene-3 in a sample which contains cells which comprises steps of:
- 35



-131-

- (a) obtaining total RNA from the cell, contacting the RNA so obtained with a labelled nucleic acid molecule of claim 92 under hybridizing conditions; and
- 5 (b) determining the presence of RNA hybridized to the molecule, thereby detecting the expression of the Prostate Tumor Inducing Gene-3 in the sample.
- 10 99. A method of detecting expression of a Prostate Tumor Inducing Gene-3 in tissue sections which comprises steps of
- (a) contacting the tissue sections with a labelled nucleic acid molecule of claim 95 under
- 15 hybridizing conditions; and
- (b) determining the presence of RNA hybridized to the molecule, thereby detecting the expression of the Prostate Tumor Inducing Gene-3 in tissue sections.
- 20 100. An isolated mammalian nucleic acid molecule of claim 89 operatively linked to a promoter of RNA transcription.
- 25 101. A vector which comprises the isolated mammalian nucleic acid molecule of claim 89.
102. A plasmid of claim 101.
- 30 103. The plasmid of claim 102 designated PTI-3 having ATCC Accession No. 97022.
104. A virus of claim 102.
- 35 105. A DNA virus of claim 102.

-132-

106. An RNA virus of claim 102.
107. A retrovirus of claim 102.
- 5 108. A host vector system for the production of a polypeptide having the biological activity of a mammalian Prostate Tumor Inducing Gene-3 protein which comprises the vector of claim 96 and a suitable host.
- 10 109. A host vector system of claim 108, wherein the suitable host is a bacterial cell, insect cell, plant cell or mammalian cell.
- 15 110. A method of producing a polypeptide having the biological activity of a mammalian Prostate Tumor Inducing Gene-3 protein which comprises growing the host cells of the host vector system of claim 109 under suitable conditions permitting production of
- 20 the polypeptide and recovering the polypeptide so produced.
111. A mammalian cell comprising the vector of claim 101.
- 25 112. Purified mammalian Prostate Tumor Inducing Gene-3 protein.
113. A polypeptide encoded by the isolated mammalian nucleic acid molecule of claim 89.
- 30 114. A method to produce antibody using the mammalian Prostate Tumor Inducing Gene-1 protein of claim 112 or 113.
- 35 115. An antibody capable of binding specifically to the

-133-

mammalian Prostate Tumor Inducing Gene-1 protein of claim 112 or 113.

116. A monoclonal antibody of claim 115.

5

117. A pharmaceutical composition comprising an antibody of claim 114 or 115 and a pharmaceutically acceptable carrier.

10

118. A therapeutic agent comprising an antibody of claim 114 or 115 and a cytotoxic agent.

119. A therapeutic agent of claim 118, wherein the cytotoxic agent is either a radioisotope or toxin.

15

120. An immunoassay for measuring the amount of a mammalian Prostate Tumor Inducing Gene-3 protein in a biological sample comprising steps of:

20

a) contacting the biological sample with the antibody of claim 114 or 115 under the condition permitting the formation of a complex with said antibody and the mammalian Prostate Tumor Inducing Gene-3 protein, and

25

b) measuring the amount of said complex, thereby measuring the amount of the Prostate Tumor Inducing Gene-3 protein in said biological sample.

30

121. A method of inactivating oncogenic transformation of cells comprising inactivating the expression of the 5'-UTR of Prostate Tumor Inducing Gene-1, -2 or -3.

35

122. A method of claim 121, wherein the inactivation is carried out by using the antisense oligonucleotide having a sequence capable of inactivating the

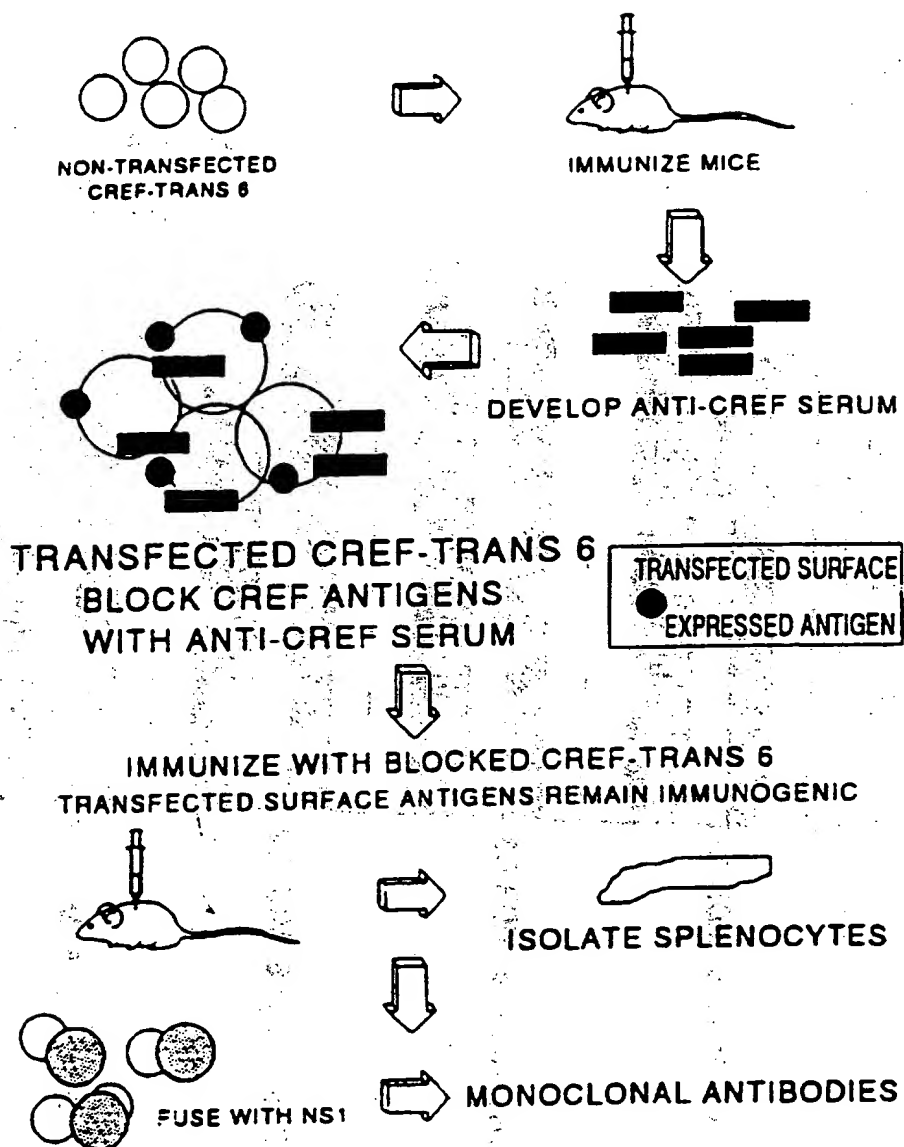
-134-

complete or a portion of the 5'-UTR of Prostate Tumor Inducing Gene-1 sequence.

- 5 123. A method of claim 121, wherein the inactivation is carried out by using the antisense oligonucleotide having a sequence capable of inactivating the complete or a portion of the 5'-UTR of Prostate Tumor Inducing Gene-2 sequence.
- 10 124. A method of claim 121, wherein the inactivation is carried out by using the antisense oligonucleotide having a sequence capable of inactivating the complete or a portion of the 5'-UTR of Prostate Tumor Inducing Gene-3 sequence.
- 15 125. A method of claim 122, 123, or 124 wherein the antisense oligonucleotide is introduced to cells by a retroviral vector.
- 20 126. A method of claim 122, 123, or 124 wherein the antisense oligonucleotide is introduced to cells by a DNA virus vector.
- 25 127. A method of claim 122, 123, or 124 wherein the antisense oligonucleotide is introduced to cells by an RNA virus vector.

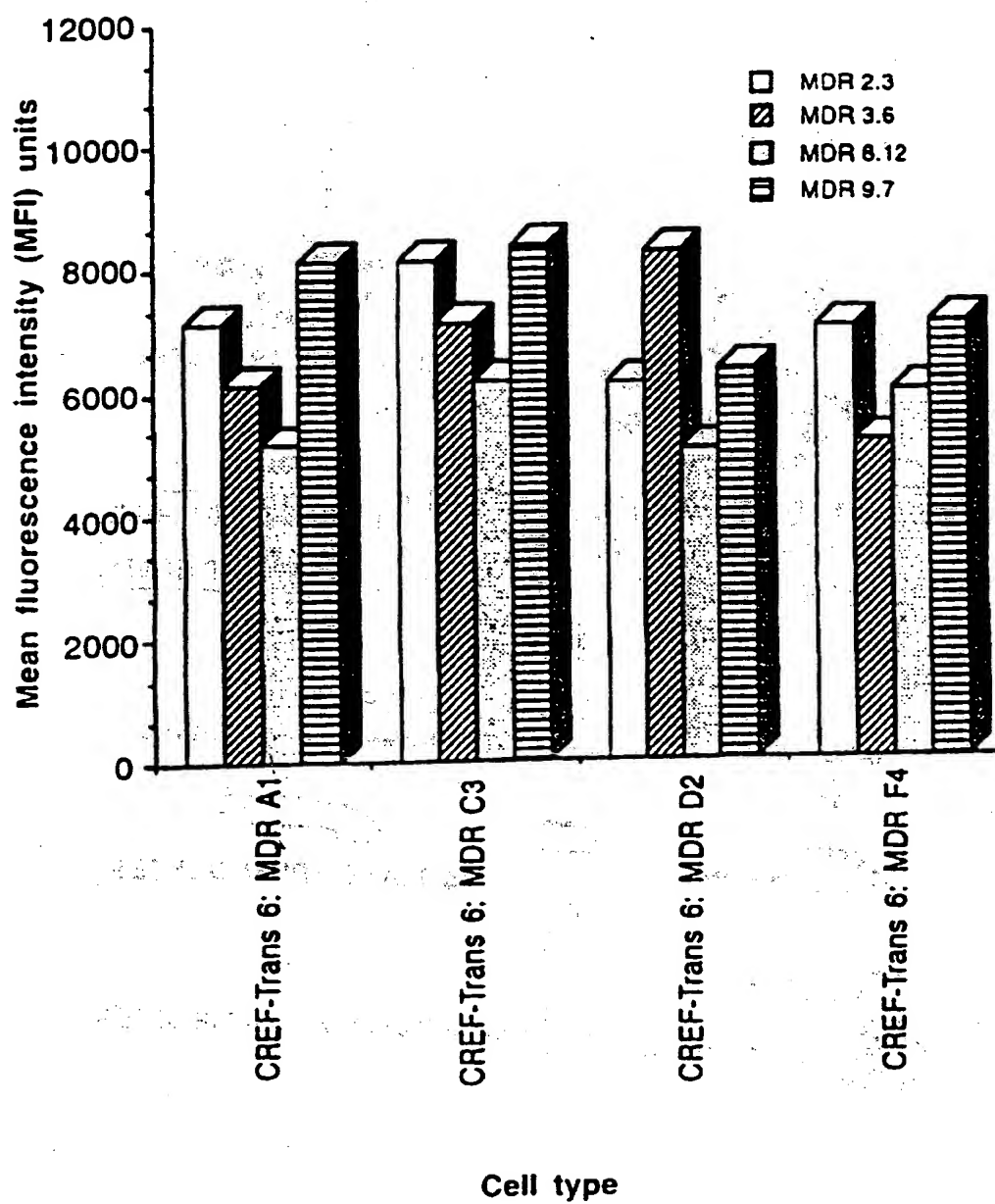
1/28

FIGURE 1



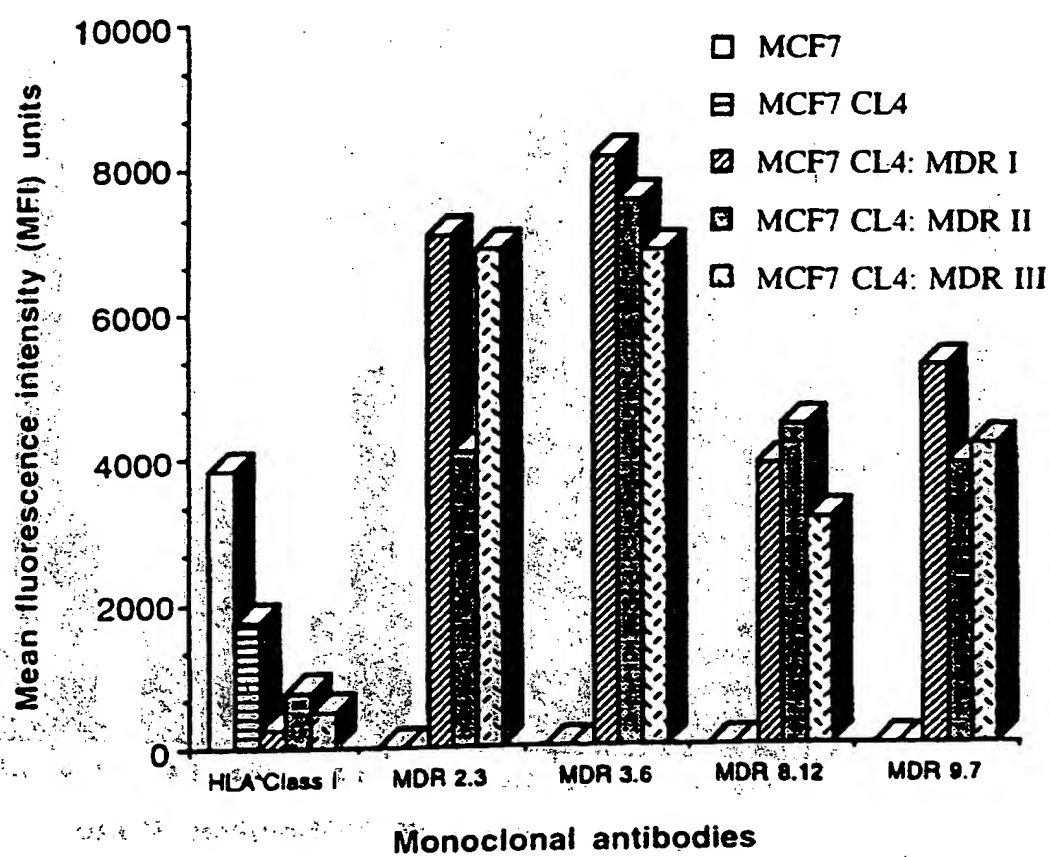
2/28

FIGURE 2



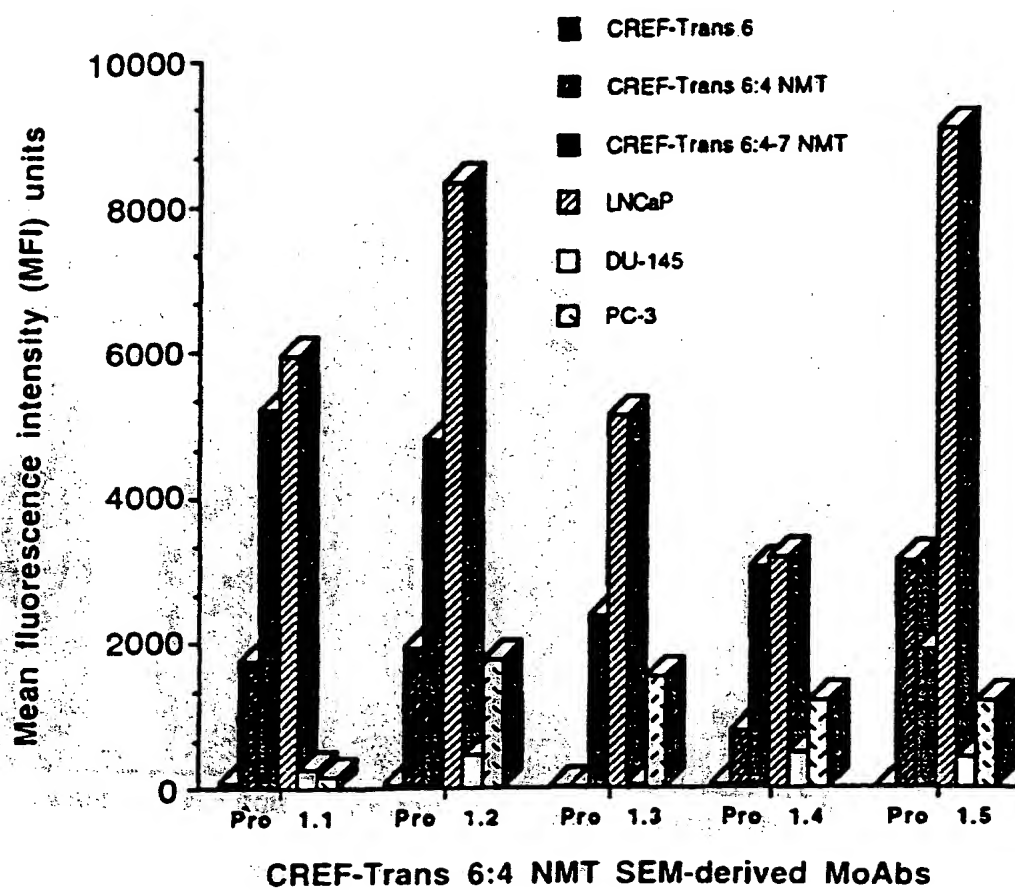
3/28

FIGURE 3



4/28

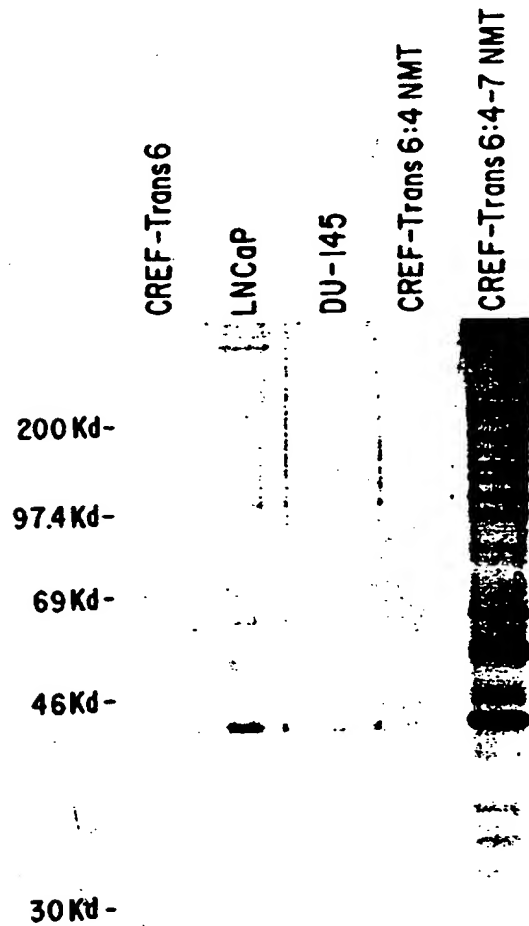
FIGURE 4





5/28

FIGURE 5



6/28

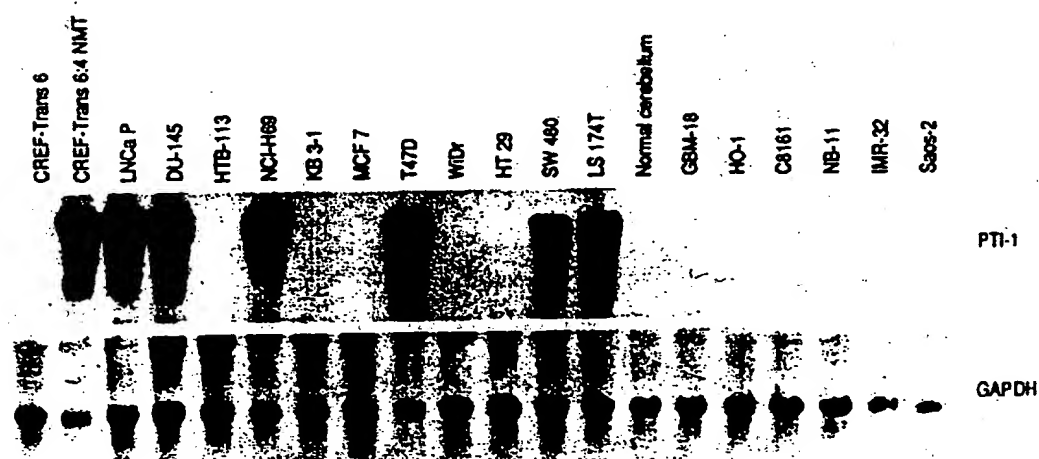
**FIGURE 6**

CREF-Trans 6  
CREF-Trans 6:4 NMT



7/28

FIGURE 7



**FIGURE 8A**

1 gataacgaatcataaatactcatagatgtagtctgagtagggcgggggcccgtaaacccctcgaatctcgccgccaccaccggtaagge 90  
91 taatactaaacagacacccgatagtagaactagtagccgtaggggaaggtgaaaagaaccgagagggsgagtagaataagattc:gaacc 179  
180 att:acttacaag:gggtccalltact:acaaggttcagagcacgttaag:tgatggcgatact:tcgagtagggcgccgag:taag: 27:  
272 taatacgcaaggttaagcagaaaaaagcgaggccgtagggaaccgagtcgtaalaggcgacittagatattgcatataccggaat 361  
362 caggtagatctccatgagcagggtgaagcttaggttaaaactaaagtgaggagaccgagtagtactgataaaagtcgccgtaggact: 45:  
452 gtaggtagtgtagaataccaatgaaaccgtgagtagctgtgtctctcgaatagcttttagggctagcgtagtagtattgttaaggggg 543  
544 tagagcactggaatgtaggaatggcgccatctagctgactgactataaacaactccgaataccattaaaataagcIATG CAG TCG 629  
M Q S  
630 GAA CGT GGT ATC ACC ATT GAT ATC TCC TTG TGG AAA TTT GAG ACC AGC AAG TAC TAT 686  
E R G I T I D I S L W K F E T S K Y Y  
687 GTG ACT ATC ATT GAT GCC CCA GGA CAC AGA GAC TTT ATC CAA AAC ATG ATT ACA GG 742  
V T I I D A P G H R D F I Q N M I T G  
743 G ACC TCT CAG GCT GAC TGT GCT GTC CTG ATT GTT GCT GCT GGT GTT GGT GAA TTT GAA G 80:  
G S Q A D C A V L I V A A G V G E F E  
802 CT GGT ATC TCC AAG AAT GGG CAG ACC CGA CAG CAT GCC CTT CTG GCT TAC ACA CTG GG 858  
A G I S K N G Q T R E H A L L A Y I L G  
859 T GTG AAA CAA CTA ATT GTC GGT GTT AAC AAA ATG GAT TCC ACT GAG CCA CCC TAC 914  
V K Q L I N G V N K M D S T E P P Y  
915 AGC CAG AAG AGA TAT GAG GAA ATT GTT AAG GAA GTC AGC ACT TAC ATT AAG AAA 968  
S Q K R Y E E I V K E V S T Y I K K  
969 ATT GGC TAC AAC CCC GAC ACA GTA GCA TTT GTG CCA ATT TCT GGT TGG AAT GGT GAC 1025  
I G Y N P D T V A F V P I S G W N G D  
1026 AAC ATG CTG GAG CCA AGT OCT AAC ATG CCT TGG TTC AAG GGA TGG AAA GTC ACC CGT 1082  
N M L E P S A N M P W F K G W K V T R  
1083 AAG GAT GGC AAT GCC AGT GGA ACC ACG CTG CTT GAG GCT CTG GAC TGC ATC CTA CCA 1139  
K D G N A S G T T L L E A L D C I L P  
1140 CCA ACT CGT CCA ACT GAC AAG CCC TTG GGC CTG CCT CTC CAG GAT GTC TAC AAA ATT 1196  
P T R P T D K P L Q L P L Q D V Y K I  
1197 GGT GGT ATT GGT ACT GTT CCT GTT GGC CGA GTG GAG ACT GGT GTT CTC AAA CCC GGT 1253  
G G I G T V P V G R V E T G V L K P G  
1254 ATG GTG GTC ACC TTT CGT CCA GTC AAC GTT ACA ACG GAA GTA AAA TCT GTC GAA ATG 1310  
M V V T F Q P V N V T T E V K S V E M  
1311 CAC CAT GAA GCT TTG GGT GAA GCT CTT CCT GGG GAC AAT GTG GGC TTC AAT GTC AAG 1367  
H H E A L Q E A L P G D N V G F N V K  
1368 AAT GTG TCT GTC AAG GAT GTT CGT CGT GGC AAC GTT OCT GGT GAC AGC AAA AAT GAC 1424  
N V S V K D V R R G N V A G D S K N D  
1425 CCA CCA ATG GAA GCA GCT GGC TTC CCT GCT CAG GTG ATT ATC CTG AAC CAT CCA GGC 1481  
P P M E A A G F P A Q V I I L N H P Q  
1482 CAA ATA AGC GCC GGC TAT GCC CCT GTA TTG GAT TGC CAC ACG GCT CAC ATT GCA TGC 1538  
Q I S A G Y A P V L D C H T A H I A C  
1539 AAG TTT GCT GAG CTG AAG GAA AAG ATT GAT CGC CGT TCT GGT AAA AAG CTG GAA 1592  
K F A E L K E K I D R R S G K K L E  
1593 GAT GGC CCT AAA TTC TTG AAG TCT GGT GAT GCT GCC ATT GTT GAT GGT CCT GGC 1649  
D G P K F L K S G D A A I V D M V P G  
1650 AAG CCC ATG TGT GTT GAG AGC TTC TCA GAC TAT CCA CCT TTG GGC TGC TTT GCT GTT 1706  
K P M C V E S F S D Y P P L G Q F A V  
1707 CGT GAT ATG AGA CAG ACA GTT GCG GTG GGT GTC ATC AAA GCA GTG GAC AAG AAG GCT 1763  
R D M R Q T V A V G V I K A V D K K A  
1764 GCT GGA GCT GGC AAG GTC ACC AAG TCT GCC CAG AAA GCT CAG AAG GCT AAA TGA 1817  
A G A G K V T K S A Q K A Q K A K ter  
1818 atattatccctaatatccctcccccactcttaatcag:ggtaggaagaccgggttcagaaac:gtttgttcaattggccatttaagtttag: 190:  
1910 agtaaaagacttggttaatgataacaatgcatcgtataaaccttcagaaaggaaggaagttttgttgaccacgttggtttctttttgc 1996  
1997 gttgggcagttttaaagttattag:tttttaaaatcagtaacttttaatggaaacaacttgacccccaaatttgacacagaa:tttgggaccca: 1089  
2090 taataaagggttaactggggaataaataaataaataa 2123

9/28

## FIGURE 8B

(E)1 MGKEKTHINIVVIGH 15

(E)16 YDSGKSTHGHILVKCGGIDKRTTEKEKEAAEMGKSEKYAWVLDKCLKAER 67  
(P)1 MQS 3

(E)68 ERGITIDISLWKFETSKYYVTIDAPGHRDFIKNMITGTSQADCAVLIVAAGV 120  
(P)4 ERGITIDISLWKFETSKYYVTIDAPGHRDFIQNMITGTSQADCAVLIVAAGV 56

(E)121 GEFEAGISKNGQTRHALLAYTLGVKQLVGVNKNMDSTEPPYSQKRYEEIVKE 173  
(P)57 GEFEAGISKNGQTRHALLAYTLGVKQLVGVNKNMDSTEPPYSQKRYEEIVKE 109

(E)174 VSTYKKIGYNPDTVAFVPISGWNNDNMLEPSANMPWFKGWKVKTRKDGNA 223  
(P)110 VSTYKKIGYNPDTVAFVPISGWNNDNMLEPSANMPWFKGWKVKTRKDGNA 159

(E)224 SGTILLEALDCILPPTPTDKPLRLPLQDVYKIGIGITVPVGRVETGVLPKPGM 276  
(P)160 SGTILLEALDCILPPTPTDKPLGLPLQDVYKIGIGITVPVGRVETGVLPKPGM 212

(E)277 VVTFAPVNVTTTEVKS VEMHHEALSEALPGDNVGFNVKNVSVKDVRRGNV 325  
(P)213 VVTFGPVNVTTTEVKS VEMHHEALGEALPGDNVGFNVKNVSVKDVRRGNV 261

(E)326 AGDSKNDPPMEAAAGFTAQVILNHPGQISAGYAPVLDCHTAHLACKFAELK 376  
(P)262 AGDSKNDPPMEAAAGFPAQVILNHPGQISAGYAPVLDCHTAHLACKFAELK 312

(E)377 EKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPPLGCFVVRD 428  
(P)313 EKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPPLGCFVVRD 364

(E)429 MRQTVAVGVTKAVDKKAAGAGKVTKSAQKAK 462  
(P)365 MRQTVAVGVTKAVDKKAAGAGKVTKSAQKAK 398

10/28

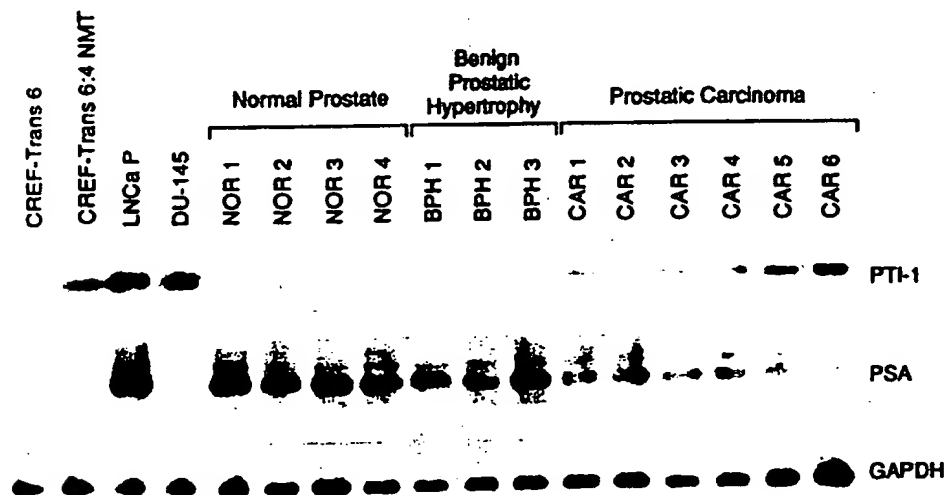
FIGURE 8C

Human	Amino Acid	K (100)	R (247)	A (281)	S (300)	T (341)	R (423)
EF-1 $\alpha$	Codon	AAA	CGC	GCT	AGT	ACT	CGC
	Nucleotide	A	C	C	A	A	C

PTI-1	Amino Acid	Q (36)	G (183)	G (217)	G (236)	P (277)	C (359)
	Codon	CAA	GGC	GGT	GGT	CGT	TGC
	Nucleotide	C	G	G	G	C	T

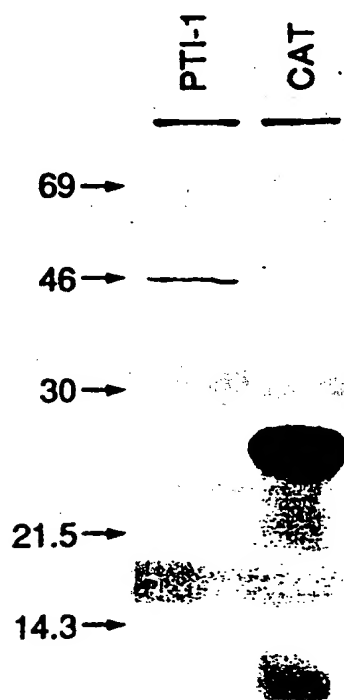
11/28

FIGURE 9



12/28

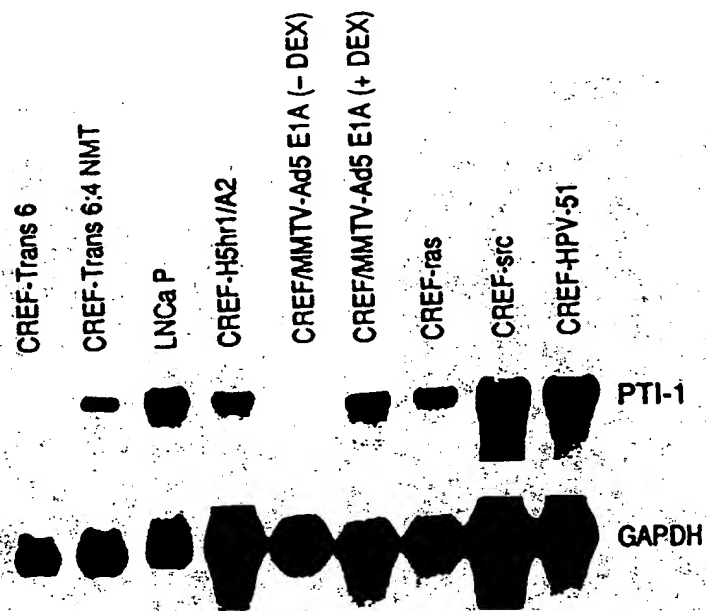
FIGURE 10





13/28

FIGURE 11



14/28

FIGURE 12B

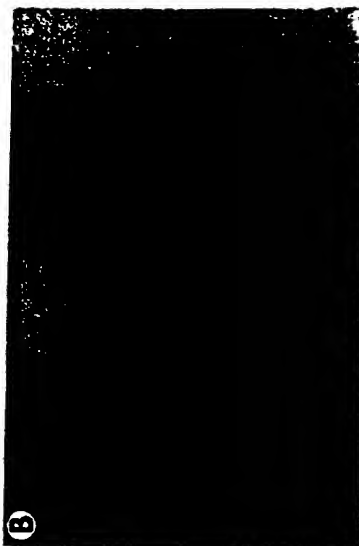


FIGURE 12D



FIGURE 12A



FIGURE 12C



15/28

**FIGURE 13A**

1 CCGACCGAGC TCCGTTGCAT TTTGATGAAT CCATAGTCAA ATTAGCGAGA  
51 CACGTTGCCA ATTGAAACAT CTTAGTAGCA ACAGGAAAAG AAAATAAATA  
101 ATGATTTTCGT CAGTAGTGGC GAGCGAAAGC GAAAGAGCCC AAACCTGTAA  
151 AGGGGGGTTG GTAGGACATC TTACATTGAG TTACAAAATT TTATGATAGT  
201 AGAAGAAGTT GGGAAAGCTT CAACATAGAA GGTGATATTC CTGTATACGA  
251 AATCATAAAA TCTCATAGAT GTATCCTGAG TAGGGCGGGG TACGTGAAAC  
301 CCTGTCTGAA TCTGCCCCGG ACCACCCGTA AGGCTAAATA CTAATCAGAC  
351 ACCGATAGTG AACTAGTACC GTGAGGGAAA GGTGAAAAGA ACCCGAGAGG  
401 GGAGTGAAAT AGATTCTGAA ACCATTTACT TACAAGTAGT CAGAGCACGT  
451 TAAAGTGTGA TGGCGTACAT CTTGCAGTAT GGGCCGGCGA GTTATGTAA  
501 TATGCAAGGT TAAGCACGAA AAAAGCGGAG CCGTAGGGAA ACCGAGTCTG  
551 AATAGGGCGA CTTTAGTATA TTGGCATATA CCCGAAACCA GGTGATCATC  
601 CATGAGCAGG TTGAAGCTTA GGTAAACTA AGTGGAGGAC CGAACCGTAG  
651 TACGCTAAAA AGTGCCCGGA TGACTTGTGG ATAGTGGTGA AATTCCAATC  
701 GAACCTGGAG ATAGCTGGTT CTCTTCGAAA TAGCTTTAGG GCTAGCGTAT  
751 AGTACTGTTT AATGGGGGTA GAGCACTGAA TGTGGAATGG CGGCATCTAG  
801 CTGTACTGAC TATAATCAAA CTCCGAATAC CATTAAAATT AAGCTATGCA  
851 GTCGGAACGT GGGTGATAAC GTCCACGCTC GCGAGGGAAA CAACCCAGAT  
901 CCGTCAGCTA AGGTCCCAAA ATTGTGTAA GTGAGAAAGG TTGTGGAGAT  
951 TTCATAAACA ACTAGGAAGT TGGTTTAGAA GCAGCCACCT TTAAAGAGT  
1001 GCGTAATTGC TCACTAGTCA AGAGATCTTG CGCCAATAAT GTAACGGGAC  
1051 TCAAACACAA TACCCAAGCT ACGGGCACAT TATGTGCGTT AGGAGAGCGT  
1101 TTTAATTTTCG TTGAAGTCAG ACCGTGAGAC TGGTGGAGAG ATTAAGGTT  
1151 CGAGAATGCC GGCATGAGTA ACGATTCGAA GTGAGAATCT TCGACGCCTA  
1201 TTGGGAAAGG TTTCTGGGC AAGGTTCTCC ACCCAGGGTT AGTCAGGGCC  
1251 TAAGATGAGG CAGAAATGCA TAGTCGATGG ACAACAGGTT AATATTCCTG

16/28

**FIGURE 13B**

1301 TACTTGGTAA AAGAATGATG GAGTGACGAA AAAGGATAGT TCTACCACTT  
1351 CCACTATGTC CTATCAATAG GAGCTGTATT TGGCATCATA GGAGGCTTCA  
1401 TTCACTGATT TCCCCTATTG TCAGGCTACA CCCTAGACCA AACCTACGCC  
1451 AAAATCCATT TCACTATCAT ATTCATCGGC GTAAATCTAA CTTTCTTCCC  
1501 ACAACACTTT CTCGGCCTAT CCGGAATGAC CCGACCCGAC GTTACTCGGA  
1551 CTACCCCGAT GCATACACCA CATGAAACAT CCTATCATCT GTAGGCTCAT  
1601 TCATTICTCT AACAGCAGTA ATATTAATAA TTTTCATGAT TTGAGAAGCC  
1651 TTCGCCTTCG AAGCGAAAAG TCCTAATAGT AGAAGAACCC TCCATAAACC  
1701 TGGAGTGACT ATATGGATGC CCCCACCCTA CCTCACATTC GAAGAACCCG  
1751 TATACATAAA ATCTAGACAA AAAAGGAAGG AAGTGAACGC CCCACAAAAA  
1801 AAAAAAAAAA AAAAAAAAAA

17/28

**FIGURE 14A**

1 AACTAAGTGG AGGACCGAAC CGTAGTACGC TAAAAAGTGC CCGGATGACT  
51 TGTGGATAGT GGTGAAATTC CAATCGAACC TGGAGATAGC TGGTTCTCTT  
101 CGAAATAGCT TTAGGGCTAG CGTATAGTAT TGTTTAATGG GGGTAGAGCA  
151 CTGAATGTGG AATCGGCGGC ATCTAGCTGT ACTGACTATA ATCAAACCTCC  
201 GAATACCATT AAAATTAAGC TATGCAGTCG GAACGTGGGT GATAACCTCC  
251 ACTCTCGCGA GGGAAACAAC CCAGATCGTC AGCTAAGGTC CCAAATTTGT  
301 GTTAAGTGAG AAAGGTTGTG AGATTTCATA ACAAACCTAGG AAGTTGGCTT  
351 AGAAGCAGCC ACCTTTTAAA GAGTGCGTAA TTGCTCACTA GTCAAGAGAT  
401 CTTGCGCCAA TAATGTAACG GGA CTCAAAC ACAATACCGA AGCTACGGGC  
451 ACATTATGTC GGTTAGGAGA GCGTTTAAAT TTCGTTGAAG TCAGACCGTG  
501 AGACTGGTGG AGAGATTAAA AGTTCGAGAA TGCCCGGCAT GAGTAACGAT  
551 TCGAAGTGAG AATCTTCGAC GCCTATTGGG AAAGGTTTCC TGGGCAAGGT  
601 TCGTCCACCC AGGGTTAGTC AGGGCCTAAG ATGAGGCAGA AATGCATAGT  
651 CGATGGACAA CAGGTTAATA TTCCTGTACT TGGTAAAAGA ATGATGGAGT  
701 GACGAAAAAG GATAGTTCTA CCACTTACTG GATTGTGGGG TAAGCAACAA  
751 GAGAGTTATA TAGGCAAATC CGTATAGCAT AATCTTGAGT TGTGATGCAT  
801 AGTGAAGACT TCGGTCGAGT AACGAATTGA ATCGATTTC TGTTCCTAAG  
851 AAAAGCTTCT AGTGTTAATT TTTTATCAAC CTGTACCGAG AACGAACACA  
901 CGTTCCCAAG ATGAGTATTC TAAGGCGAGC GAGAAAACCA ATGTTAAGGA  
951 ACTCTGCAAA ATAACCCCGT AAGTTCGCGA GAAGGGGCGC CTATTTTAA  
1001 TAGGCCACAG AAAATAGGGG GGCAACTGTT TATCAAAAAC ACAGCTCTCT  
1051 GCTAAGTTGT AAAACGACGT ATAGAGGGTG AAGCCTGCCC AGTCCCGAAG  
1101 TTAAACGGAG ATGTTAGCTT ACGCAAAGCA TTAAAGTGAA GCCCGGGTGA  
1151 ACGGCGGCCG TAACTATAAC GGTCTTAAGG TAGCGAAATT CCTTGTC AAC  
1201 TAATTATTGA CCTGCACGAA AGGCGCAATG ATCTCCCTAC TGTCTCAACA  
1251 TTGGA CTGCG TGAAATTATG GTACCAGTGA AAACGCAGGT TACCCGCATC

18/28

**FIGURE 14B**

1301 AAGACGAAAA GACCCCGTGG AGCTTTACTA TAACTTCGTA TTGAAAATTG  
1351 GTTTAGCATG TGTAGGATAG GCGGGAGACT TTGAAGCTGG GACGCTAGTT  
1401 CTAGTGGAGT CAACCTTGAA ATACCACCCT TGCTAAATTG ATTTTCTAAC  
1451 CCGTTCCCCT TATCTGGAAG GAGACAGTGC GTGGTGGGTA GTTTGACTGG  
1501 GCGGTCGCCT CCTAAAGTGT AACGGAGGCG TTCAAAGCTA CACTCAATAT  
1551 GGTCAGAAAC CATATGCAGA GCACAAAGGT AAAAGTGTGG TTGACTGCAA  
1601 GACTTACAAG TCGAGCAGGT GCGAAAGCAG GACTTAGTGA TCCGGCGGTA  
1651 CATTGTGGAA TGGCCGTCGC TCAACGGATA AAAGTCACCC CGGGGATAAC  
1701 AGGCTAATCT TCCCCAAGAG ATCACATCGA CGGGAAGGTT TGGCACCTCG  
1751 ATGTCGGCTC ATCGCATCCT GGAGCTGGAG TCGGTTCCAA GGGTTTGCTG  
1801 TTCGCCAATT AAAGCGGTAC GTGAGCTGGG TTCAGAACGT CGTGAGACAG  
1851 TTCGGTCCTC CACTTAGTT

19/28

## FIGURE 15A

- 1 CGGCACGAGC GGCACGAGAG AAGAGACTCC AATCGACAAG  
AAGCTGGAAA
- 51 AGAATGATGT TGTCTTAAA CAACCTACAG AATATCATCT  
ATAACCCGGT
- 101 AATCCCGTTT GTTGGCACCA TTCCTGATCA GCTGGATCCT  
GGAACCTTGA
- 151 TTGTGATACG TGGGCATGTT CCTAGTGACG CAGACAGATT  
CCAGGTGGAT
- 201 CTGCAGAATG GCAGCAGCGT GAAACCTCGA GCCGATGTGG  
CCTTTCATT
- 251 CAATCCTCGT TTCAAAAGGG CCGGCTGCAT TGTTTGCAAT  
ACTTTGATAA
- 301 ATGAAAAATG GGGACGGGAA GAGATCACCT ATGACACGCC  
TTTCAAAAGA
- 351 GAAAAGTCTT TTGAGATCGT GATTATGGTG CTGAAGGACA  
AATTCCAGGT
- 401 GGCTGTAAAT GGAAAACATA CTCTGCTCTA TGGCCACAGG  
ATCGGCCCAG
- 451 AGAAAATAGA CACTCTGGGC ATTTATGGCA AAGTGAATAT  
TCACTCAATT
- 501 GGTTTTAGCT TCAGCTCGGA CTTACAAAGT ACCCAAGCAT  
CTAGTCTGGA
- 551 ACTGACAGAG ATAGTTAGAG AAAATGTTCC AAAGTCTGGC  
ACGCCCCAGC
- 601 TTAGCCTGCC ATTCGCTGCA AGGTTGAACA CCCCCATGGG  
CCCTGGACGA
- 651 ACTGTCGTCG TTCAAGGAGA AGTGAATGCA AATGCCAAAA  
GCTTTAATGT
- 701 TGACCTACTA GCAGGAAAAT CAAAGGATAT TGCTCTACAC  
TTGAACCCAC
- 751 GCCTGAATAT TAAAGCATTT GTAAGAAATT CTTTCTTCA  
GGAGTCCTGG
- 801 GGAGAAGAAG AGAGAAATAT TACCTCTTTC CCATTAGTC  
CTGGGATGTA

20/28

**FIGURE 15B**

- 851 CTTTGAGATG ATAATTATT GTGATGTTAG AGAATTCAAG  
GTTGCAGTAA
- 901 ATGGCGTACA CAGCCTGGAG TACAAACACA GATTTAAAGA  
GCTCAGCAGT
- 951 ATTGACACGC TGGAAATTAA TGGAGACATC CACTTACTGG  
AAGTAAGGAG
- 1001 CTGGTAGCCT ACCTACACAG CTGCTACAAA AACCAAAATA  
CAGAATGGCT
- 1051 TCTGTGATAC TGGCCTTGCT GAAACGCATC TCACTGGTCA  
TTCTATTGTT
- 1101 TATATTGTTA AAATGAGCTT GTGCACCATT AGGTCCTGCT  
GGGTGTTCTC
- 1151 AGTCCTTGCC ATGACGTATG GTGGTGTCTA GCACTGAATG  
GGGAAACTGG
- 1201 GGGCAGCAAC ACTTATAGCC AGTTAAAGCC ACTCTGCCCT  
CTCTCCTACT
- 1251 TTGGCTGACT CTTCAAGAAT GCCATTCAAC AAGTATTTAT  
GGAGTACCTA
- 1301 CTATAATACA GTAGCTAACA TGTATTGAGC ACAGATTTTT  
TTTGGTAAAT
- 1351 CTGTGAGGAG CTAGGATATA TACTTGGTGA AACAAACCAG  
TATGTTCCCT
- 1401 GTTCTCTTGA GCTTCGACTC TTCTGTGCGC TACTGCTGCG  
CACTGCTTTT
- 1451 TCTACAGGCA TTACATCAAC TCCTAAGGGG TCCTCTGGGA  
TTAGTTATGC
- 1501 AGATATTAAA TCACCCGAAG AACTAACTT ACAGAAGACA  
CAACTCCTTC
- 1551 CCCAGTGATC ACTGTCATAA CCAGTGCTCT GCCGTATCCC  
ATCACTGAGG
- 1601 ACTGATGTTG ACTGACATCA TTTTCTTTAT CGTAATAAAC  
ATGTGGCTCT
- 1651 ATTAGCTGCA AGCTTTACCA AGTAATTGGC ATGACATCTG  
AGCACAGAAA
- 1701 TTAAGCCAAA AAACCAAAGC AAAACAAATA CATGGTGCTG  
AAATTAACCT



21/28

## FIGURE 15C

- 1751 GATGCCAAGC CCAAGGCAGC TGATTCTGT GTATTGAAC  
TTACCCGAAA
- 1801 TCAGAGTCTA CACAGACGCC TACAGAAGTT TCAGGAAGAG  
CCAAGATGCA
- 1851 TTCAATTTGT AAGATATTTA TGGCCAACAA AGTAAGGTCA  
GGATTAGACT
- 1901 TCAGGCATTC ATAAGGCAGG CACTATCAGA AAGTGTACGC  
CAACTAAGGG
- 1951 ACCCACAAAG CAGGCAGAGG TAATGCAGAA ATCTGTTTTG  
TTCCCATGAA
- 2001 ATCACCAATC AAGGCCTCCG TTCTTCTAAA GATTAGTCCA  
TCATCATTAG
- 2051 CAACTGAGAT CAAAGCACTC TTCCACTTTA CGTGATTAAA  
ATCAAACCTG
- 2101 TATCAGCAAG TTAAATGGTT CCATTCTGT GATTTTCTA  
TTATTGAGG
- 2151 GGAGTTGGCA GAAGTTCCAT GTATATGGGA TCTTTACAGG  
TCAGATCTTG
- 2201 TTACAGGAAA TTTCAAAGGT TTGGGAGTGG GGAGGGAAAA  
AAGCTCAGTC
- 2251 AGTGAGGATC ATTCCACATT AGACTGGGGC AGAACTCTGC  
CAGGATTTAG
- 2301 GAATATTTTC AGAACAGATT TTAGATATTA TTTCTATCCA  
TATATTGAAA
- 2351 AGGAATACCA TTGTCAATCT TATTTTTTTA AAAGTACTCA  
GTGTAGAAAT
- 2401 CGCTAGCCCT TAATCTTTT CCAGCTTTTC ATATTAATGT  
ATGCAGAGTC
- 2451 TCACCAAGCT CAAAGACACT GGTGGGGGT GGAGGGTGCC  
ACAGGGAAAG
- 2501 CTGTAGAAGG CAAGAAGACT CGAGAATCCC CCAGAGTTAT  
CTTCTCCAT
- 2551 AAAGACCATC AGAGTGCTTA ACTGAGCTGT TGGAGACTGT  
GAGGCATTTA
- 2601 GGAAAAAAT AGCCCACTCA CATCATTCCT TGTAAGTCTT  
AAGTTCATT

22/28

## FIGURE 15D

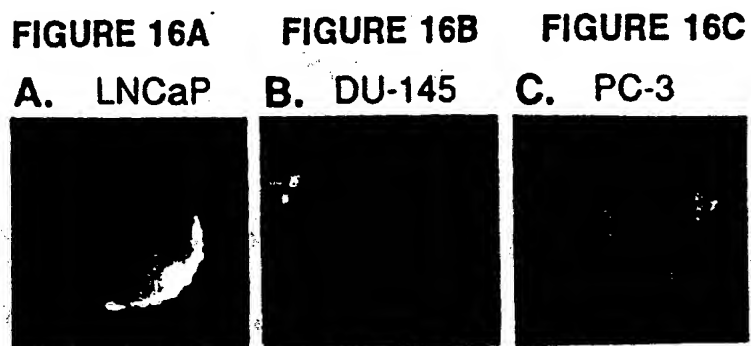
- 2651 TCATTTTACG TGGAGGAAAA AAATTTAAAA AGCTATTAGT  
ATTTATTAAT
- 2701 GAATTTTACT GAGACATTTC TTAGAAATAT GCACTTCTAT  
ACTAGCAAGC
- 2751 TCTGTCTCTA AAATGCAAGT TGGCCTTTTG CTGCCCACAT  
TTCTGCATTA
- 2801 AACTTCTATA TTAGCTTCAA AGGCTTTTAA TCTCAATGCG  
AACATTCTAC
- 2851 GGGATGTTCT TAGATGCCTT TAAAAAGGGG GCAAGATCTA  
ATTTTATTG
- 2901 AACCCCTCACT TTCCAACCTT CACCATGACC CAGTACTAGA  
GATTAGGGCA
- 2951 CTTCAAAGCA TTGAAAAAAA TCTACTGATA CTTACTTTCT  
TAGACAAGTA
- 3001 GTTCTTAGTT AACCACCAAT GGAAGTGGGT TCATTCTGAA  
TCCTGGAGGA
- 3051 GCTTCCTCGT GCCACCCAGT GTTCTGGGC CCTCTGTGTG  
AGCAGCCAGG
- 3101 TGTGAGCTGT TTTAGAAGCA GCGTGTGCC TTCATCTCTC  
CCGTTTCCCA
- 3151 AAAGAACAAA GGATAAAGGT GACAGTCACA CTCCTGGGTT  
AAAAAAGCA
- 3201 TTCCAGAACC ACTTCTCTTT ATGGGCACAA CAACAAAGAA  
GCTAAGTTCG
- 3251 CCTACCCAAA TGAAAGTAGG CTTTACAGTC AAGTACTTCT  
GTTGATTGCT
- 3301 AAATAACTTC ATTTTCTTGA AATAGAGCAA CTTTGAGTGA  
AATCTGCAAC
- 3351 ATGGATACCA TGTATGTAAG ATACTGCTGT ACAGAAGAGT  
TAAGGCTTAC
- 3401 AGTGCAAATG AGGCGTCAGC TTTGGGTGCT AAAATTAACA  
AGTCTAATAT
- 3451 TATTACCATC AATCAGGAAG AGATAATAAA TGTTTAAACA  
AACACAGCAG
- 3501 TCTGTATAAA AATACGTGTA TATTTACTCT TTCTGTGCAC  
GCTCTATAGC

23/28

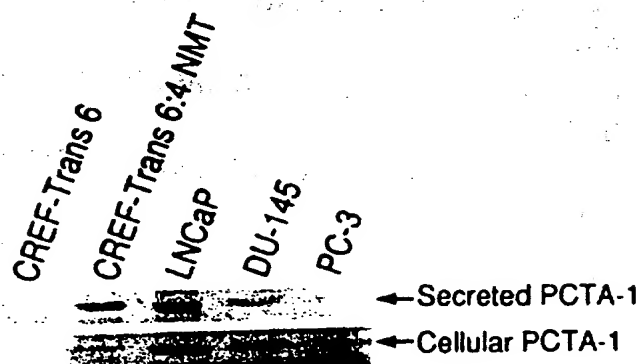
**FIGURE 15E**

- 3551 ATAGGCAGGA GAGGCTTATG TGGCAGCACA AGCCAGGTGG  
GGATTTTGT
- 3601 AAGAAGTGAT AAAACATTG TAAGTAATCC AAGTAGGAGA  
TATTAAGGCA
- 3651 CCAAAAGTAA CATGGCACCC AACACCCAAA AATAAAAATA  
TGAAATATGA
- 3701 GTGTGAACTC TGAGTAGAGT ATGAAACACC ACAGAAAGTC  
TTAGAAATAG
- 3751 CTCTGGAGTG GCTCTCCCAG GACAGTTTCC AGTTGGCTGA  
ATAGTCTTTT
- 3801 GGCAGTGATG TTCTACTTCT TCACATTCAT CTAAAAAAA  
AAAAAAAAA

24/28



**FIGURE 16D**      Secreted and Cellular PCTA-1



25/28

FIGURE 17A

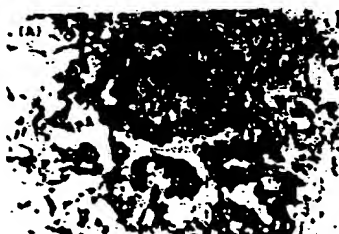


FIGURE 17B



FIGURE 17C



FIGURE 17D



FIGURE 17E

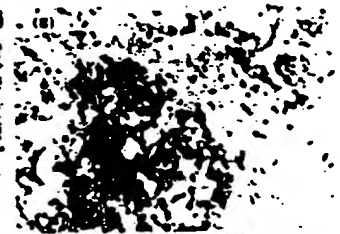


FIGURE 17F

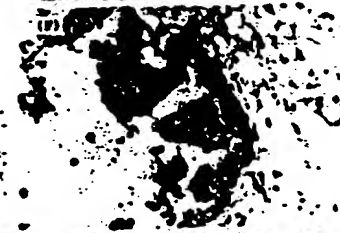


FIGURE 17G



FIGURE 17H



26/28

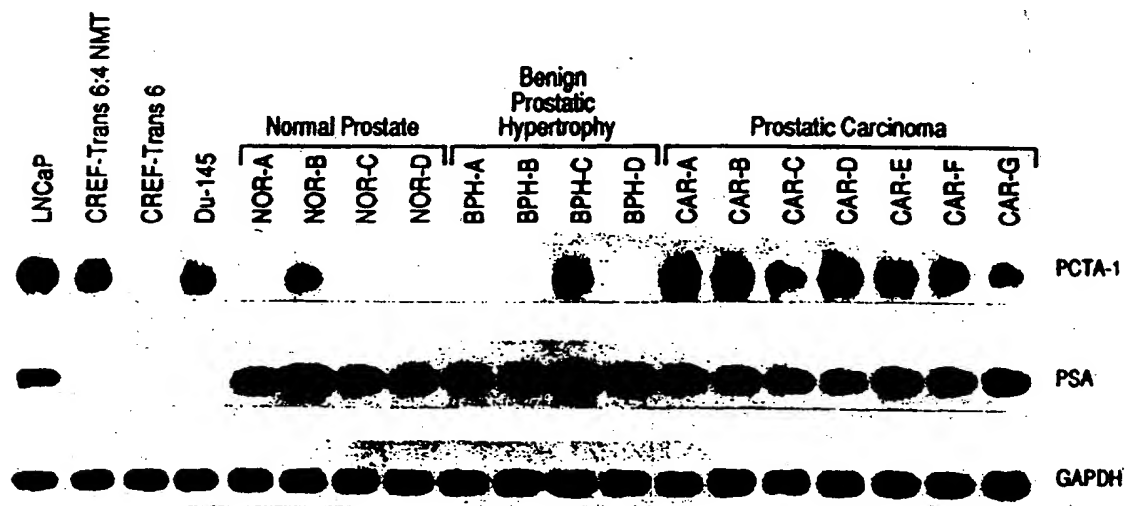
FIGURE 18A

54 ATG ATG TTG TCC TTA AAC AAC CTA CAG AAT ATC ATC TAT AAC CCG GTA ATC CCG TTT GTT  
 M L S L N N L Q N I I I Y N P V I P F V  
 114 GGC ACC ATT CCT GAT CAG CTG GAT CCT GGA ACT TTG ATT GTG ATA CGT GGG CAT GTT CCT  
 G T I P D Q L D P G T L I V I R G H V P  
 174 AGT GAC GCA GAC AGA TTC CAG GTG GAT CTG CAG AAT GGC AGC GTG AAA CCT CGA GCC  
 S D A D R F Q V D L Q N G S S V K P R A  
 234 GAT GTG GCC TTT CAT TTC AAT CCT CCG TTT CAA AGG GCC GGC TGC ATT GTT TGC AAT ACT  
 D V A F H F N P R F K R A G C I V C N T  
 294 TTG ATA AAT GAA AAA TGG GGA CCG GAA GAG ATC ACC TAT GAC AGC CCT TTC AAA AGA GAA  
 L I N E K W G R E E I T Y D T P F K R E  
 354 AAG TCT TTT GAG ATC GTG ATT ATG GTG CTG AAG GAC AAA TTC CAG GTG GCT GTA AAT GGA  
 K S F E I V I M V L K D K F Q V A V N G  
 414 AAA CAT ACT CTG CTC TAT GGC GAC AGG ATC GGC CGA GAG AAA ATA GAC ACT CTG GGC ATT  
 K H T L L Y G H R I G P E K I D T L G I  
 474 TAT GGC AAA GTG AAT ATT CAC TCA ATT GGT TTT AGC TTC AGC TCG GAC TTA CAA AGT ACC  
 Y G K V N I H S I G F S F S D L Q S T  
 534 CAA GCA TCT AGT CTG GAA CTG ACA GAG ATA GTT AGA GAA AAT GTT CCA AAG TCT GGC ACG  
 Q A S S L E L T E I V R E N V P K S G T  
 594 CCC CAG CTT ACC CTG CCA TTC GCT GCA AGG TTG AAC ACC CCC ATG GGC CCT GGA CGA ACT  
 P Q L S L P F A A R L N T P M G P G R T  
 654 GTC GTC GTT CAA GGA GAA GTG AAT GCA AAT GCC AAA AGC TTT AAT GTT GAC CTA CTA GCA  
 V V V Q G E V N A N A K S F N V D L L A  
 714 GGA AAA TCA AAG GAT ATT GCT CTA CAC TTG AAC CCA CGC CTG AAT ATT AAA GCA TTT GTA  
 G K S K D I A L H L N P R L N I K A F V  
 774 AGA AAT TCT TTT CTT CAG GAG TCC TGG GGA GAA GAG AGA AAT ATT ACC TCT TTC CCA  
 R N S F L Q E S W G E E R N I T S F P  
 834 TTT AGT CCT GGG ATG TAC TTT GAG ATG ATA ATT TAT TGT GAT GTT AGA GAA TTC AAG GTT  
 F S P G M Y F E M I I Y C D V R E F K V  
 894 GCA GTA AAT GGC GTA CAC AGC CTG GAG TAC AAA CAC AGA TTT AAA GAG CTC AGC AGT ATT  
 A V N G V H S L E Y K H R F K E L S I  
 954 GAC ACG CTG GAA ATT AAT GGA GAC ATC CAC TTA CTG GAA GTA AGG AGC TGG TAG  
 D T L E I N G D I H L L E V R S W +



28/28

FIGURE 19





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00307

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04

US CL : 536/23.5, 24.31

According to International Patent Classification (IPC) or to both national classification and IPC.

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5, 24.31; 435/6, 69.1, 91.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,314,996 A (WRIGHT JR.) 24 May 1994, column 2, lines 53-55, and column 12, line 46, bridging to column 13, line 10.	1-8
Y	US 5,227,471 A (WRIGHT JR.) 13 July 1993, column 3, lines 15-17, and column 13, lines 8-39.	1-8
A	US 5,162,504 A (HOROSZEWICZ) 10 November 1992, see entire document.	1-8
Y	SU ET AL. Transfer of a dominant-acting tumor-inducing oncogene from human prostatic carcinoma cells to cloned rat embryo fibroblast cells by DNA-transfection. Anticancer Research. 1992, Volume 12, pages 297-304, especially page 298.	1-8

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 APRIL 1996

Date of mailing of the international search report

8 MAY 1996

Name and mailing address of the ISA/US  
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Authorized officer

BRADLEY L. SISSON

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00307

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	SHEN ET AL. Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. Proceedings of the National Academy of Sciences, United States of America, July 1995, Volume 92, pages 6778-6782, especially page 6779.	1-8
A	NAKHLA ET AL. Characterization of ALVA-41 cells, a new human prostatic cancer cell line. Steroids. October 1994, Volume 59, pages 586-589, see entire document.	1

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00307

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00307

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN

Search Terms: prostate, carcinoma, tumor, antigen, nucleic acid, DNA, RNA, Columbia University, R Shen, P B Fisher

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1 and 6-8, drawn to nucleic acid; claims 2-4 and 11, drawn to DNA; claim 5, drawn to RNA; and claims 9 and 10, drawn to a method of detecting expression of Prostate Carcinoma Tumor Antigen Gene-1.

Group II, claim 12, drawn to vector; claims 13 and 18 drawn to a plasmid; claims 14-17, drawn to viral vectors; claims 19 and 20, drawn to host vector system; claim 21, drawn to recombinant method of producing Prostate Carcinoma Tumor Antigen Gene-1 polypeptide; claim 22, drawn to method of transforming cells; and claim 23, drawn to transformed cells.

Group III, claims 24 and 25, drawn to Prostate Carcinoma Tumor Antigen Gene-1 polypeptide.

Group IV, claims 26, 27, 29-31, and 39, drawn to antibodies, including monoclonal antibodies (claim 27) as well as antibodies labeled with a cytotoxic agent (claims 30, 31, and 39).

Group V, claim 28, drawn to competitive inhibitor of antibody which binds Prostate Carcinoma Tumor Antigen Gene-1 polypeptide.

Group VI, claims 32-35, drawn to method of measuring the amount of mammalian Prostate Carcinoma Tumor Antigen Gene-1 protein.

Group VII, claim 36, drawn to method to determine whether a patient carries a cancer with a metastatic potential.

Group VIII, claim 37, drawn to method for determining whether a compound is capable of inhibiting the expression or activity of Prostate Carcinoma Tumor Antigen Gene-1 protein.

Group IX, claim 38, drawn to method for determining whether a compound is capable of specifically binding to the Prostate Carcinoma Tumor Antigen Gene-1 protein.

Group X, claim 40, drawn to a method of treating cancer with metastatic potential.

Group XI, claims 41, 46-49, and 52, drawn to nucleic acid; claims 42-44, drawn to DNA; and claim 45, drawn to RNA.

Group XII, claims 50 and 51, drawn to method of detecting expression of Prostate Tumor Inducing Gene-1.

Group XIII, claim 53, drawn to vector containing Prostate Tumor Inducing Gene-1; claims 54 and 55, drawn to plasmids containing said Prostate Tumor Inducing Gene-1; claim 56, drawn to viral vectors containing Prostate Tumor Inducing Gene-1; claim 57, drawn to DNA viral vectors containing Prostate Tumor Inducing Gene-1; claims 58 and 59, drawn to RNA viral vectors containing Prostate Tumor Inducing Gene-1; claims 60, 61, and 63, drawn to host vector system comprising Prostate Tumor Inducing Gene-1; and claim 62, drawn to recombinant method of making Prostate Tumor Inducing Gene-1 protein.

Group XIV, claims 64 and 65, drawn to Prostate Tumor Inducing Gene-1 protein.

Group XV, claim 66, drawn to method to produce an antibody to Prostate Tumor Inducing Gene-1 protein; and claims 67-71 drawn to said antibodies.

Group XVI, claim 72, drawn to immunoassay for Prostate Tumor Inducing Gene-1 protein.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00307

Group XVII, claims 73 and 78-81, drawn to nucleic acid having nucleotide sequence of Prostate Tumor Inducing Gene-1.

Group XVIII, claims 73 and 78-81, drawn to nucleic acid having the sequence of Prostate Tumor Inducing Gene-2; claims 74-76, drawn to DNA of Prostate Tumor Inducing Gene-2; and claim 77, drawn to RNA of Prostate Tumor Inducing Gene-2.

Group XIX, claim 82, drawn to vectors comprising Prostate Tumor Inducing Gene-2; claims 83 and 84, drawn to plasmids containing Prostate Tumor Inducing Gene-2; claim 85, drawn to viral vectors containing Prostate Tumor Inducing Gene-2; claim 86, drawn to DNA viruses containing Prostate Tumor Inducing Gene-2 and claims 87 and 88, drawn to RNA viruses containing Prostate Tumor Inducing Gene-2.

Group XX, claims 89, 94-97, and 100, drawn to nucleic acid containing Prostate Tumor Inducing Gene-3; claims 90-92, drawn to DNA containing Prostate Tumor Inducing Gene-3; and claim 93, drawn to RNA containing Prostate Tumor Inducing Gene-3.

Group XXI, claims 98 and 99, drawn to method of detecting Prostate Tumor Inducing Gene-3 expression.

Group XXII, claim 101, drawn to vector containing Prostate Tumor Inducing Gene-3; claims 102 and 103, drawn to plasmid containing Prostate Tumor Inducing Gene-3; claim 104, drawn to viral vector containing Prostate Tumor Inducing Gene-3; claim 105, drawn to DNA viral vector containing Prostate Tumor Inducing Gene-3; claims 106 and 107, drawn to RNA viral vectors containing Prostate Tumor Inducing Gene-3; claims 108, 109, and 111, drawn to host vector system containing Prostate Tumor Inducing Gene-3; and claim 110, drawn to recombinant method of producing Prostate Tumor Inducing Gene-3 protein.

Group XXIII, claims 112 and 113, drawn to Prostate Tumor Inducing Gene-3 protein.

Group XXIV, claim 114, drawn to a method of making antibodies to Prostate Tumor Inducing Gene-3 protein; and claims 115-119, drawn to said antibodies.

Group XXV, claim 120, drawn to an immunoassay for measuring Prostate Tumor Inducing Gene-3 protein.

Group XXVI, claim 121, drawn to a method of inactivating oncogenic transformation of cells comprising inactivation of expression of the 5'-UTR of Prostate Tumor Inducing Gene-1, Prostate Tumor Inducing Gene-2, or Prostate Tumor Inducing Gene-3; claim 122, drawn to antisense therapy for Prostate Tumor Inducing Gene-1; claim 123, drawn to antisense therapy for Prostate Tumor Inducing Gene-2; claim 124, drawn to antisense therapy for Prostate Tumor Inducing Gene-3; claim 125, drawn to the method of claim 121 where a viral vector is used; claim 126, drawn to the method of claim 121, where a DNA viral vector is used; and claim 127, drawn to the method of claim 121 where a RNA viral vector is used.

The inventions listed as Groups I-XXVI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The groups are directed to different nucleic acid sequences which correspond to different genes and their respective different uses, different means for detection, and different products which are derived therefrom. Specifically, the different nucleic acid sequences, including DNA and RNA, which are claimed correspond to Prostate Carcinoma Tumor Antigen Gene-1; Prostate Tumor Inducing Gene-1; Prostate Tumor Inducing Gene-2; and Prostate Tumor Inducing Gene-3. Accordingly, the claimed inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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